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Characterization of the immune response in mice treated with materials toxic for macrophages

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CHARACTERIZATION OF THE IMMUNE RESPONSE IN MICE TREATED WITH MATERIALS TOXIC FOR MACROPHAGES

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ABSTRACT

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Characterization of the Immune Response in Mice Treated With Material Toxic for Macrophages (127 pp.)

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RML mice were treated with Min-U-Sil or carrageenan, both of which were toxic for macrophages. After 6 h, the mice were immunized intravenously (i.v.) with either lipopolysaccharide (LPS) or sheep red blood cells (SRBC). The kinetics of the subsequent antibody responses were determined by hemagglutination. Treatment with these toxins prior to immunization did not suppress the primary antibody response to either antigen nor did it impair priming for a secondary antibody response. In fact, enhanced primary antibody responses were obtained in some macrophage impaired mice. The outcome depended on the dose of antigen used to challenge mice and the time of immunization after macrophage impairment. Macroglobulin (19 S) antibody production was found to be prolonged in mice immunized during macrophage impairment. Secondary type antibody responses to LPS could be elicited with one dose of LPS, if given 21 days after macrophage impairment. From these results, a regulatory role for the macrophage in the afferent limb of the humoral immune response was proposed.

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ABBREVIATIONS

α	phagocytic index corrected for differences in body and organ weight
AMG	anti-macrophage globulin
ARU	antigen reactive unit
B-cell	bursa-equivalent lymphocyte
EMEM	Eagles minimal essential medium
FFNMS	fresh frozen normal mouse sera
i.p.	intraperitoneal
i.v.	intravenous
K	phagocytic index
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LPS*	chromium^{51} labeled lipopolysaccharide
2-ME	2-mercaptoethanol
MUS	Min-U-Sil
PBS	phosphate buffered saline
PEC	peritoneal exudate cells
PFC	direct plaque-forming cell
PMN	polymorphonuclear leukocyte
RES	reticuloendothelial system
RML	Rocky Mountain Laboratory
S	Svedberg units
SRBC	sheep red blood cells
T-cell	thymus derived lymphocyte

CHAPTER I

INTRODUCTION

Recent investigations of the initiation and development of the immune response through studies of cellular interactions have led to an increased awareness of the complexity of the immune system. Attempts to define the role of these cellular interactions in the initiation of the immune response have become an active area of research in modern immunology. Both cell-mediated immunity and antibody production appear to be direct results of previous cellular collaboration. The term "antigen reactive unit" (ARU), has been coined by Pross and Eidnger (64) to define the minimum complex of cells and factors necessary to produce a detectable immune response in reaction to antigenic stimulation. A full understanding of the ARU is essential in delineating the immune response at the molecular level, as well as offering a means of practical immunoregulation.

Macrophages are perhaps the most controversial cellular component of the ARU. Since the time of Metchnikoff, the role of macrophages in a host's resistance to disease has remained a controversial area of research. Early studies into the understanding of antibody formation, apparently indicated that the macrophages were responsible for both the uptake of antigen and synthesis of antibody (cf 45). As a consequence, a great part of the research in

immunology was devoted to evaluating the uptake of antigen by macrophages, in addition to studying the physiology of macrophages. Once it was recognized that lymphocytes and plasma cells were the cells involved in antibody synthesis (cf 45), the interest in macrophages subsided momentarily. However, the extensive investigations of the mechanisms of lymphocyte interactions and the molecules released from immunocompetent lymphocytes have regenerated an interest in determining the precise role(s) that macrophages may play in the development of optimal immune responses (35). The macrophage now appears to be a key cell in the efferent limb of cell-mediated immunity (57, 90). However, its involvement as an essential cellular component of the afferent limb of humoral immunity or even of cell-mediated immunity has remained controversial (83).

Numerous mechanisms have been proposed for the function(s) of macrophages as possible auxillary cells in the afferent limb of the immune response. Some investigators view macrophages as a scavenger cell, whose primary purpose is to engulf antigen and break it down into immunologically nonreactive products (24, 61). In this role, macrophages would appear to act as a safeguard of the immune system protecting the host from antigenic overdose (13). It has been shown by Fujiwara and Cinader (34), and by others (18) that a reduction in the macrophage population of a strain of mice which were

resistant to induction of tolerance led to the development of tolerance when these animals were subsequently challenged with tolerogen.

Other investigators felt that, in addition to the role of scavenger cells, macrophages also might play a key role in the afferent limb of humoral immunity (26, 33, 35, 38, 56, 63). Since macrophages themselves were unable to produce antibodies, it had been suggested that they might transmit some form of information about the antigen to the lymphoid cells and in the process "educate" them. It was hypothesized that these "educated" lymphoid cells then would be capable of generating a specific antibody response. Fishman (30) and other investigators (1, 10, 39) have suggested that macrophages produced antigen-specific RNA which coded for the specific antibody to be produced. Feldmann and Palmer (27) proposed that macrophages degraded the antigen into fragments, which were capable of stimulating directly the lymphoid cells. Other investigators (3, 23, 32, 85, 86, 87) have presented data which suggested that the macrophage enhanced antigenicity by "processing" the antigen and modifying it to a highly immunogenic form.

Unanue and co-workers (88) have proposed that small quantities of antigen resisted phagocytosis and tended to persist on the surface of macrophages. Unanue (88) and Nossal (59) theorized that the immune response operated

best when antigen was concentrated and focused by mononuclear phagocytes. Still others have proposed that macrophages released nutritive and regulatory factors that modulated lymphocyte responsiveness to antigenic stimulation (19). Many substances have been found which were released by macrophages in what appears to be a true secretory process. These included numerous enzymes, complement proteins and a number of factors defined only by their biological activity on other cells (15, 16, 84, 89). Recently, it has been demonstrated that macrophages could be replaced by their own culture supernatant fluids or by 2-mercaptoethanol when tissue culture systems were employed to measure antibody responses to certain antigens (19, 44, 51). The possibility that 2-mercaptoethanol might stimulate directly the transformation of mouse lymphoid cells into blast cells had been evaluated. However, there was no evidence that 2-mercaptoethanol was a mitogen; rather, it appeared to act by maintaining lymphocyte viability and function.

Several investigations have focused on the role of macrophages in vivo in the immune response. In general, these studies have involved macrophage transfer experiments (75, 78, 82) or have employed the use of agents which either blockade the reticuloendothelial system (RES) (29, 70, 72) or were selectively toxic for macrophages (8, 52, 78, 81). Studies of the immune response to antigen

administered at the time of RES blockade (8, 29, 71) have determined that the extent and duration of blockade was dependent upon the concentration and type of blocking agent employed. Sabet and co-workers (71, 72) have shown a 20 fold depression in the number of antibody-forming cells when the blocking agents were given a day or two before subsequent challenge with antigen. Studies in which carrageenan, a high molecular weight polygalactose, was employed as a macrophage toxin indicated that the generation of the immune response was dependent upon the route of subsequent antigenic dose (8). If antigen was administered i.v. when carrageenan was given i.p. there was no change in the antibody response. However, when antigen was injected i.p. a decreased antibody response resulted.

In other studies, the immunogenicity of free antigen versus antigen associated with macrophages has been compared. Generally, it was found that antigens which were phagocytized poorly in vivo by macrophages induced at least a 10 fold increase in the immune response when presented as the macrophage-associated form rather than the free form (55, 77, 85). However, a different situation was encountered with antigens that were strongly phagocytized by macrophages in vivo. In this situation the free form of the antigen induced a 2 fold increase in the immune response when compared to a comparable amount of the macrophage-associated antigen (21, 82).

Katz and Unanue (47) have shown that the increased immunogenicity of macrophage-associated antigen may not necessarily be due to the processing of antigen by macrophages. In experiments when hapten-protein conjugates were used, it was found that fibroblasts were comparable to macrophages in their capacity to present antigen in an increased immunogenic form. These authors postulated that T-lymphocytes were triggered best by antigen bound to any cell and that after this step, the activated T-lymphocytes regulated the triggering of B-lymphocytes.

On the other hand, there were other situations in which macrophages were shown to have an immunologically suppressive effect. A decreased immune response was obtained when antigen was injected into the peritoneal cavity of mice which had been stimulated previously so that the ascites fluid contained a high concentration of macrophages (31). If iodoacetate, an inhibitor of phagocytosis, was injected into the peritoneal cavity prior to the injection of antigen, the immune response was restored (31).

Although the experimental protocols and model systems employed were highly variable, some general characterizations about the immune response in vivo could be made from these studies. In order to demonstrate immunological responsiveness in vivo, immunocompetent lymphocytes were necessary. That is to say when antigen-associated macrophages were transferred into immunologically tolerant mice,

or into mice in which lymphoid function had been impaired by x-irradiation, there was no detectable immunological response (21, 55). Investigators also have demonstrated up to a 5 fold increase in antibody titer, when antigen was associated with viable syngenic macrophages as opposed to non-viable, allogeneic or xenogenic macrophages (53, 82). Thus, these data suggested that viable genetically compatible macrophages might also be necessary for immunocompetency in vivo. But, in contrast to the above studies, it has been found also that the immune response could be inhibited partially in vivo, when normal macrophages were injected simultaneously with macrophages containing antigen (78, 87).

Thus, despite this great amount of experimental data and proposed mechanisms, the role of macrophages in the generation of antibodies has remained unresolved. Neither the use of in vitro nor in vivo systems appeared to have proven a definitive role for the macrophages in the afferent limb of humoral immunity. Conflicting evidence as well as conflicting conclusions continued to be elicited by various investigations. It was still unclear whether macrophages were absolutely essential in the induction of the immune response or just facilitated antigen-stimulated lymphocyte transformation.

Statement of Purpose

The purpose of this study was to determine if a reduction in the number of sessile macrophage as well as a reduction of their function would alter the subsequent antibody response. More specifically, there were two areas of concern in this study: (1) to determine the extent and duration of macrophage impairment induced by various treatment; and (2) to characterize the immune status of the mouse at the time of maximum macrophage impairment.

The phagocytic activity of the RES in mice was characterized following varying regimens of treatment with crystalline silica and carrageenan. The kinetics of the primary antibody response were characterized in macrophage-impaired mice and were compared with those of normal mice. The distribution of the antigen in the macrophage-impaired mouse was investigated also. Subsequently the ability of the macrophage-impaired mouse to prime for a secondary antibody response was evaluated. Finally, in several cases, the qualitative type of antibody engendered in the response was determined.

CHAPTER II

MATERIALS AND METHODS

Animals

All experiments were performed on 5-8 week old Swiss-Webster derived mice of both sexes obtained from the Rocky Mountain Laboratory (RML) stock, Hamilton, Montana. The mice were housed in groups of 5 and allowed food and water *ad libitum*.

Preparation of carrageenan

Calcium carrageenan in the form of Sea Kem-9^{R} was generously donated by Dr. Donald Renn (Marine Colloids, Rochland, Maine). Prior to injection, the dry carrageenan was weighed, placed in a sterile vial and resuspended to the desired concentration with warm (37°C) sterile phosphate buffered saline (PBS) (0.15 M NaCl, 0.0033 M PO_4; pH 7.2).

Preparation of silica

Silica particles, (MIN-U-SIL, Whittaker, Clark and Daniels, Inc., NY, NY) of less than 5 um in average size, were used for all *in vivo* studies. Further sizing fractionation was done according to the method of Larson (50). Briefly, 50 g of silica were suspended in 300 ml

of distilled water and subjected to ultrasonic vibrations (Bronson, Danbury, Conn.) for 30 sec. This suspension was diluted to a total volume of 1 ℓ with distilled water, placed in a 1 ℓ graduated cylinder and allowed to settle at room temperature (25°C) for 24 h. Four 250 ml aliquots were then aspirated and placed in separate centrifuge tubes. Fraction III (MUS) corresponding to the 500-750 ml portion from the top, was washed two times with distilled water and allowed to dry in a warm (60°C) oven for 48 h. It was determined by optical measurement, that MUS consisted of particles of the following sizes: 2.1 um x 2.8 um (80%), 1.4 um x 3.5 um (10%), and 1.4 um x 2.1 um (10%) (50). Prior to injection, the dry MUS was weighed, placed in a sterile vial, autoclaved and resuspended to the desired concentration with sterile PBS.

Preparation of antigens

LPS from E. coli 0113. The lipopolysaccharide (LPS) used in these studies was a phenol-water extract of Escherichia coli 0113 (Braude strain) prepared as described previously by Rudbach et al. (69).

Sheep red blood cells (SRBC). SRBC preserved in Alsever's solution were obtained from the Colorado Serum Company (Denver, CO). When prepared as an antigen for immunization, the SRBC were washed thrice in cold (4°C) PBS

and the packed cells were reconstituted to the desired concentration with cold PBS.

Collection of sera for hemagglutination assays

Whole blood was obtained from mice anesthetized with ether by exsanguination following an axillary incision. The blood was allowed to clot at room temperature for approximately 1 h and then at 4°C overnight. The serum was harvested by centrifugation (1,000 x g for 10 min), dispensed into vials, heated to 56°C for 30 min and stored at -20°C.

Quantification of humoral antibodies

Antibodies specific for the LPS determinant were measured by passive hemagglutination (67). Briefly, SRBC coated with LPS extracted from E. coli 0113 were used as indicator cells (58). For sensitization, 1 mg of LPS was dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.4) and then placed in a boiling water bath for 2 ½ h. Sensitized SRBC were prepared by combining 0.25 ml of packed, washed SRBC, 9.0 ml PBS and 1 ml of the boiled LPS. This mixture was then incubated at 37°C for 30 min with frequent mixing. The cells were then washed thrice in cold PBS and finally resuspended to a total volume of 0.5 ml with microtiter diluent (PBS containing 1% heat inactivated, SRBC-

absorbed normal rabbit sera). The sensitized SRBC were used immediately after preparation.

Antibodies specific for SRBC were measured by direct hemagglutination. SRBC were washed thrice in cold PBS and 0.25 ml of the packed SRBC were then suspended to a total volume of 50 ml in microtiter diluent. The SRBC were used immediately after preparation.

To each well of a microtiter plate (Linbro Chemical Company, Inc., New Haven, CT) 0.05 ml of microtiter diluent was added and serial two-fold dilutions of the sera were made with 0.05 ml micro-dilutors (Cooke Engineering Co., Alexandria, VA). Next, 0.05 ml of SRBC or sensitized SRBC suspended to a final concentration of 0.5% were added to each well, the plate was gently agitated, covered and placed in a humid chamber. Hemagglutination was recorded after incubation of the plates for 2 h at 25°C and overnight at 4°C.

Titers were expressed as values of x derived from the equation, $x = \log_2 (HD/2)$, where HD was the reciprocal of the highest dilution of sera which produced hemagglutination. Thus, the titer was the tube number of the end-point when the first tube contained a $\frac{1}{4}$ dilution of antiserum. Sera which gave no hemagglutination at the lowest dilution tested were arbitrarily assigned a titer of 0, ie., a dilution of $\frac{1}{2}$. All serum samples were tested in

duplicate and titers were expressed as the average titer of both tests.

Treatment of sera with 2-mercaptoethanol (2-ME)

Sera were treated with 2-ME to inactivate the hemagglutinating activity of IgM antibodies. To the first well of a microtiter plate were added 25 ul of serum, 25 ul PBS and 50 ul of 0.2 M 2-ME (Sigma Chemical Co., St. Louis, MO). This mixture was allowed to incubate at 37°C for 30 min in a sealed humid chamber. After incubation, 0.05 ml of the microtiter diluent was added to the remaining wells and the sera were diluted and assayed as described previously. These experiments were interpreted on the assumption that the residual hemagglutinating antibodies present in the sera treated with 2-ME were all of the IgG immunoglobulin class.

Phagocytic activity in vivo as measured by colloidal carbon clearance

RES function, as demonstrated by the phagocytic activity in vivo, was studied in both normal and treated mice. Data were obtained by measuring the ability of mice to clear a solution of colloidal carbon from their blood by using a modified procedure of Biozzi et al. (9). The colloidal carbon (Pelikan C11/1431a, Koh-I-Noor, Bloomsbury, NJ) was suspended to a final concentration of 10 mg/ml in

PBS containing 1% gelatin (Baker Chemical Co., Phillipsburg, NJ). The suspension was dispensed into sterile vials and frozen at -20°C until used. Test animals were weighed (Mettler Model P120, Mettler Instruments, Princeton, NJ) and then injected intravenously with 0.1 mg of prewarmed (37°C) colloidal carbon per gram body weight. The mice were anesthetized with ether (Mallinchrodt Chemical Co., St. Louis, MO) and were bled from the retro-orbital plexus at 5 and 15 min after the carbon injection. At each bleeding 0.02 ml of blood was obtained in disposable pipets (Corning, Corning, NY) that had been rinsed previously with a heparin (Riker Lab, Inc., Northridge, CA) solution of 500 units/ml. The blood samples were then lysed in 2.0 ml of a 0.1% Na_2CO_3 (Baker Chemical Co., Phillipsburg, NJ) solution and their optical densities were determined at 650 nm with a Coleman Jr. spectrophotometer (Coleman Instruments, Maywood, IL). After the 15 min blood samples were obtained, the mice were sacrificed and the wet weights of the liver and spleen were determined.

The phagocytic index (K) was determined for each mouse by the following formula (52).

$$K = \frac{\text{Log}_{10} \text{ OD at 5 min} - \text{Log}_{10} \text{ OD at 15 min}}{10 \text{ min}}$$

The phagocytic index corrected for differences in body and organ weight ($\prec$), was determined for each mouse by the

following formula (52).

$$\alpha = \sqrt[3]{\frac{K \times (\text{liver weight} + \text{spleen weight})}{\text{total body weight}}}$$

Median Lethal Dose (LD_{50}) of treated and normal mice

LD_{50} for LPS extracted from E. coli 0113 was determined for both normal and treated animals. MUS-treated mice received 10.0 mg of MUS contained in a total volume of 0.2 ml of PBS, intravenously (i.v.). These mice were then injected intraperitoneally (i.p.) with LPS contained in 0.2 ml PBS. Carrageenan-treated animals received 5.0 mg of carrageenan contained in 0.5 ml PBS i.p.; this was followed by the injection of LPS i.p. Normal mice were given PBS in the same volumes and routes as the MUS and carrageenan, followed by the injection of LPS i.p. Additional controls included mice that received only MUS or carrageenan. All experiments were performed on groups of 10 mice and were terminated after 72 h. Percent mortality of mice receiving both MUS and LPS was computed by the following formula, for each dose of LPS injected: mortality in groups receiving MUS and LPS minus mortality in groups receiving only MUS = mortality due to LPS. Mortality data were analyzed by the Formal Probit Method (4).

In vitro study of peritoneal macrophages

Peritoneal exudate cells (PEC) were stimulated by an injection of 2.0 ml of sterile 3% thioglycolate broth (Difco Laboratories, Detroit, MI) into the peritoneal cavity of mice. After 72 h, the mice were treated with either 10 mg of MUS i.v. or 5 mg of carrageenan i.p. Twenty-four hours after treatment, the mice were sacrificed and the PEC were harvested as follows. The abdominal area was cleansed with alcohol and 5.0 ml of sterile double strength Eagles Minimal Essential Media (EMEM) (Grand Island Biological Company, Grand Island, NY) was injected into the peritoneal cavity. The abdominal area was massaged, and the peritoneal fluid was aspirated and placed into a chilled (4°C) silicone (Sili-Clad, Clay Adams, Parsippany, NJ) coated tube. The harvested PEC were then assayed for their in vitro phagocytic activity by the method of Cutler (22). Briefly, the PEC were washed thrice in EMEM, counted in a hemacytometer and adjusted to a final concentration of 1.0×10^6 cells/ml with EMEM containing 10% (v/v) fresh frozen normal mouse sera (FFNMS). A 0.5 ml volume of this suspension was then dispensed onto a 22 mm^2 plastic cover slip and placed in a 37°C incubator for 30 min. After this incubation, the cover slip was rinsed vigorously thrice in EMEM to remove the non-adherent cell populations. Then 0.5 ml of EMEM containing 10% FFNMS and 2×10^6 Candida stellatoidea

cells/ml was dispensed onto the cover slip and this was followed by incubation at 37°C for 60 min. The cover slip was rinsed thrice with EMEM and then overlayed immediately with 0.5 ml of EMEM containing 10% FFNMS, and incubated at 37°C for 30 min. After this incubation, the cover slip was rinsed with PBS, air dried, stained with Wright's stain and observed under oil immersion. At least 200 cells/cover slip were counted and the percentage of cells that had phagocytized at least two yeast cells was determined.

The rest of the original cell suspension was centrifuged and resuspended in 0.2 ml of FFNMS. From this concentrated suspension slides were prepared, stained with Wright's stain and differential counts were performed.

Preparation of ^{51}Cr labeled LPS

LPS was labeled with hexavalent ^{51}Cr by a modification of the method described by Braude et al. (11). Ten mg of LPS was dissolved in 5 ml water containing 1.1 millicuries of $Na_2{}^{51}CrO_4$ (E. R. Squibb and Sons, Inc., Seattle, WA) and incubated at 37°C for 48 h. Following incubation, the solution was dialyzed against 0.001 M Na_2PO_4 (pH 7.2) at 4°C. The dialysate was changed daily until the radioactivity of the dialysate was constant for 3 days in succession. The contents of the dialysis tube were counted in order to determine the specific activity, and the labeled

LPS was diluted to a concentration of 50 ug/ml with PBS. Immediately prior to each experiment, 0.2 ml of the labeled LPS was counted to determine the specific activity.

All animals were injected i.v. with the labeled LPS contained in a total volume of 0.2 ml PBS. Radioactivity of the whole blood and organs were measured in a well-type scintillation counter (Nuclear-Chicago Corp., Des Plaines, IL) with an efficiency of approximately 5% for ^{51}Cr. To measure radioactivity, samples were placed in a glass tube and inserted into the crystal. Samples were counted and corrected for background and decay.

Test for lytic activity of MUS and carrageenan treated sera

Whole blood was collected from mice anesthetized with ether and allowed to clot for 1 h at 4°C. The sera were harvested, pooled and heat inactivated at 56°C for 30 min. Serum samples of 1 ml were added to 0.2 ml of either MUS or carrageenan; following this, 0.2 ml of a 10% SRBC suspension was added and the mixtures were incubated for 3 h at 37°C. After incubation, the samples were centrifuged (1000 x g for 10 min) to sediment the SRBC, and the supernatant fluids were removed and assayed for hemoglobin levels.

Hemoglobin levels

Hemoglobin levels were determined by a modification of

the method of Hanks et al. (41). Briefly, 10 ul of sera were added to individual tubes containing 0.5 ml of a 1% benzidine base (Fisher Scientific Co., Pittsburgh, PA) prepared in 90% acetic acid (E. I. DuPont, Inc., Wilmington, DA). To this was added 0.5 ml of a 1% hydrogen peroxide solution (J. T. Baker Chemical Co., Phillipsburg, NJ). The contents of the tubes were mixed vigorously and then incubated at 25°C for 20 min. After incubation, 5.0 ml of 10% acetic acid were added to each tube. Following an additional incubation period of 10 min at 25°C, the absorbance of the solutions were measured on a Coleman Jr. spectrophotometer at 515 nm against a 10% acetic acid blank. All samples were analyzed in duplicate.

Standard hemoglobin solutions were prepared by washing SRBC thrice with PBS. The washed packed SRBC were lysed with an equal volume of water followed by the addition of 0.5 ml toluene (Eastman Organic Chemicals, Rochester, NY). This solution was centrifuged (12,100 x g) for 10 min and the hemoglobin concentration of the supernatent fluid determined by the cyanmethemoglobin method (cf 5). Standard solutions of 5, 10, 15 and 20 mg hemoglobin/dl were prepared from the stock solution.

Collection of sera for passive transfer and enzymatic assays

Whole blood was obtained from mice and was allowed to

clot at 4°C for 1 h. The sera was harvested by centrifugation (1000 x g for 10 min) at 4°C and enzymatic assays performed immediately. Sera to be used for passive transfer were warmed to 37°C for 5 min and 1.0 ml samples were injected into the peritoneal cavity of normal mice.

Acid phosphatase (EC 3.1.3.3) determination

Acid phosphatase levels were determined on fresh pooled sera by the modified method of Roy et al. (66). Duplicate determinations of all samples were performed on a DuPont Automatic Clinical Analyzer (ACA, Model 700,000 DuPont Co., Wilmington, DE). Briefly, under acidic conditions, thymolphthalein monophosphate was hydrolyzed by phosphatase to produce thymolphthalein. The reaction was terminated with NaOH, and the absorbance read at 600 nm. The absorbance at 600 nm due to the thymolphthalein was proportional to the activity of the acid phosphatase in the sample.

Lactate dehydrogenase (EC 1.1.1.27) determination

Lactate dehydrogenase (LDH) levels were determined on fresh pooled sera by the procedure of Gay et al. (36). Duplicate determinations of all samples were performed on a DuPont Automatic Clinical Analyzer (ACA). The method can be described briefly as follows: LDH catalyzed the

oxidation of L-lactate to pyruvate with the simultaneous reduction of nicotinamide-adenine dinucleotide (NAD) to the reduced form of nicotinamide-adenine dinucleotide (NADH). Since other reactants were present in non-rate limiting quantities, the change in absorbance at 340 nm due to the appearance of NADH was directly proportional to the LDH activity.

Plaque-forming cell assay

The number of direct plaque-forming cells (PFC) specific for LPS were quantified by a hemolytic plaque assay (53). SRBC coated with LPS extracted from E. coli 0113 were employed as indicator cells. Spleens were removed from mice and a single cell suspension prepared by pressing the minced spleens through a 40 mesh stainless steel screen into cold (4°C) double strength (2X) Eagle's Medium (Grand Island Biological Co., Grand Island, NY) buffered with 1.0 M Tris-HCl. The cell suspension was washed once with 2X Eagle's, incubated for 10 min at 25°C with 0.83% ammonium chloride (NH_4Cl) to lyse the erythrocytes, and washed a second time with 2X Eagle's. Thereafter, the cells were resuspended in 3.0 ml of 1X Eagle's and appropriate plating dilutions were prepared in 1X Eagle's.

Individual tubes containing 2.0 ml of 0.8% agar

(Bacto Agar; Difco Laboratories, Inc., Detroit MI) in 1X Eagle's and 0.2 ml of 1% DEAE-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) were prepared in a 45°C water bath immediately prior to plaquing. Next, 0.2 ml of a 30% suspension of SRBC coated with LPS and 0.2 ml of the spleen cell suspension were added. The tubes were inverted thrice and the contents poured into plastic petri plates and allowed to solidify. The plates were incubated in a humid chamber for 2 h at 37°C. Following incubation. 3.0 ml of guinea pig complement (BBL Division of Bio-Quest, Cockeyville, MD) diluted 1/10 in a standard complement diluent (61) was added to each plate. The plates were then incubated for 45 min at 37°C, after which the number of plaques on the plates were counted. Duplicate plates of the same test cell suspension were made and the average number of PFC was calculated. The PFC responses of mice were expressed as the number of PFC/spleen.

Statistical analysis

When applicable, data were analyzed by the Student's *t* test (4). Differences between groups were considered significant if $p < 0.05$. Antibody titers were considered to be different if the end points were at least two tubes apart.

CHAPTER III

RESULTS

Effects of MUS on the phagocytic activity of the RES

Initial experiments were designed to determine the route of injection and amount of MUS which would produce maximum impairment of the RES phagocytic activity. Evaluation of phagocytic activity *in vivo* was based upon the ability of test mice to clear carbon particles from the peripheral blood. Figure 1 demonstrates the phagocytic activity of the RES following the i.p. injection of varying concentrations of MUS. The ability of test mice which received MUS i.p. to clear carbon from the blood was essentially the same as mice which received i.p. PBS. Likewise, multiple i.p. injections of MUS on successive days did not affect the rate of carbon clearance from the peripheral blood.

Unlike the i.p. route, a single injection of MUS i.v. produced a significant ($p < 0.05$)reduction in the rate of peripheral blood clearance of carbon (Fig. 2). A dose of 10 mg of MUS injected i.v. reduced the rate of clearance of carbon from the blood by 40%. Neither multiple injections nor increased amounts of MUS suppressed phagocytic activity. Intravenous injections of MUS in amounts greater than 20 mg were fatal to more than 50% of the experimental

Fig. 1. Phagocytic activity of the RES following i.p. injection of varying amounts of MUS. All mice were tested for RES activity 24 h after the final injection of MUS. Normal mice received an equal volume of PBS. Each value represents the arithmetic mean of 5 mice.

Symbols:

 Single injection of MUS

 MUS injected on 2 consecutive days

 MUS injected on 5 consecutive days

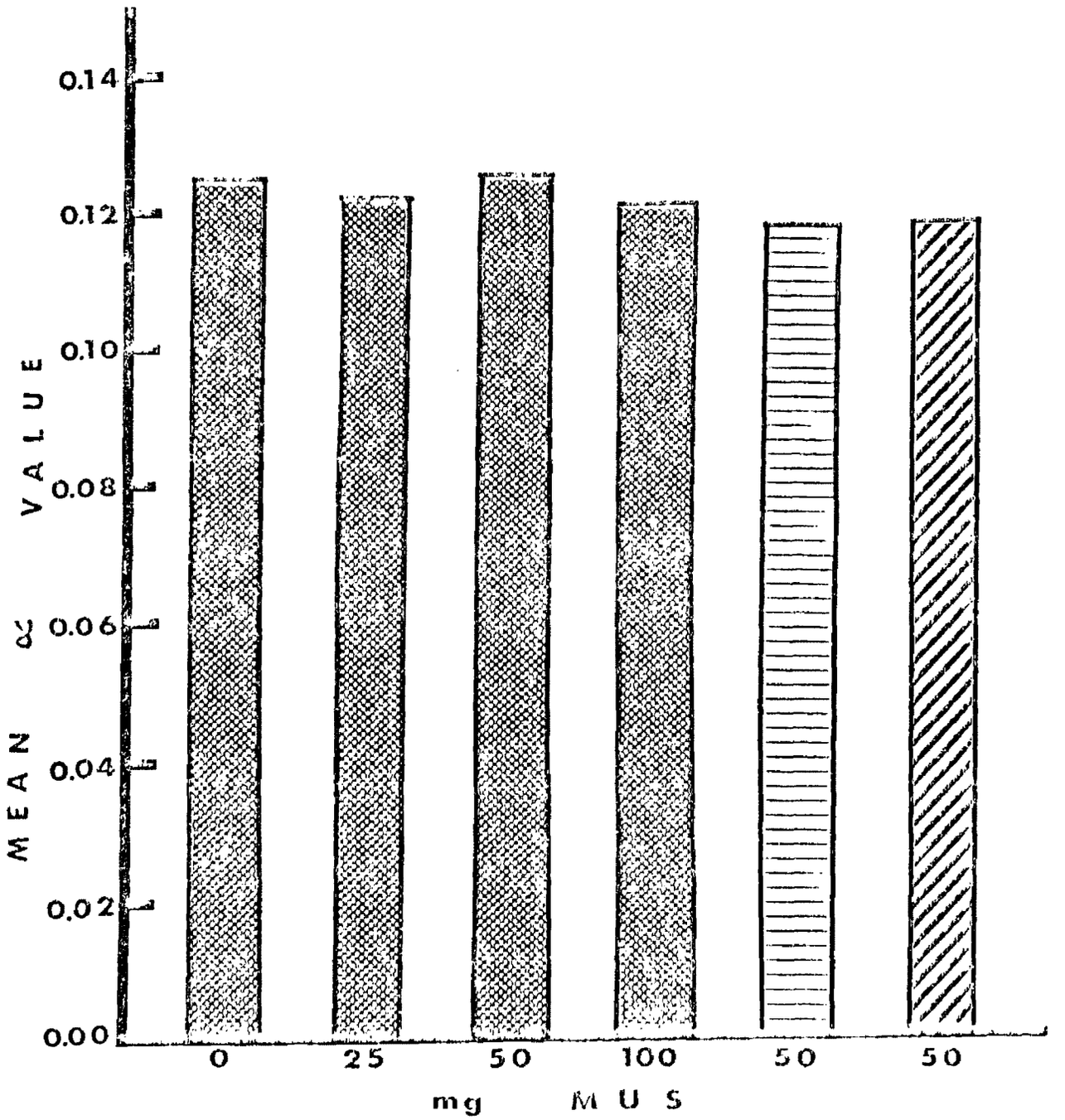
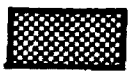
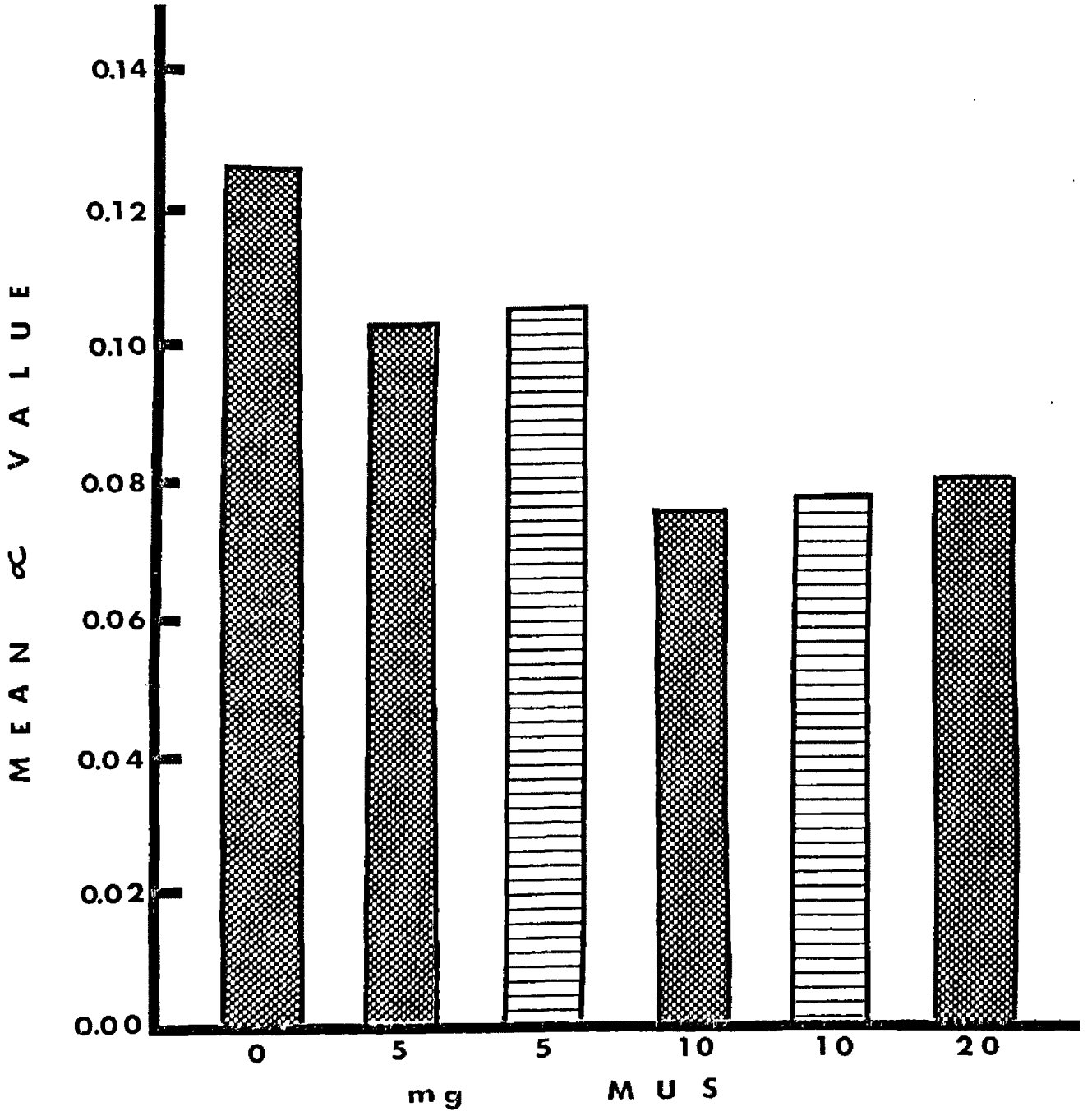


Fig. 2. Phagocytic activity of the RES following i.v. injection of varying amounts of MUS. All mice were tested for RES activity 24 h after the final injection of MUS. Normal mice received an equal volume of PBS. Each value represents the arithmetic mean of 5 mice.

Symbols:

 Single injection of MUS

 MUS injected on 2 consecutive days



animals (data now shown). Therefore, 10 mg of MUS injected i.v. was chosen as the standard regimen. Hereafter, 10 mg of MUS injected i.v. will be referred to as the standard dose of MUS.

Effects of carrageenan on the phagocytic activity of the RES

Similar experiments were designed to evaluate the ability of carrageenan to suppress RES activity. The route of injection and amount of carrageenan necessary to achieve maximum impairment of the RES were determined from the peripheral blood carbon clearance data. Figure 3 represents the phagocytic activity of the RES following the i.p. injection of carrageenan. All amounts of carrageenan administered i.p. produced a significant ($p < 0.05$) suppression of the ability of the RES to clear carbon from the blood. Maximum suppression of the phagocytic activity of the RES was obtained with 5 mg carrageenan. Doses greater than 5 mg did not further suppress RES activity but often produced ablepsia and acronecrosis.

Figure 4 represents the phagocytic activity of the RES following the i.v. injection of carrageenan. All concentrations of carrageenan injected i.v. produced significant ($p < 0.05$) suppression of RES activity, however, concentrations of 2 mg when administered i.v. were fatal to

Fig. 3. Phagocytic activity of the RES following i.p. injection of varying amounts of carrageenan. Test mice received a single i.p. injection of carrageenan and were tested for RES activity 24 h later. Normal mice received an equal volume of PBS. Each value represents the arithmetic mean of 5 mice.

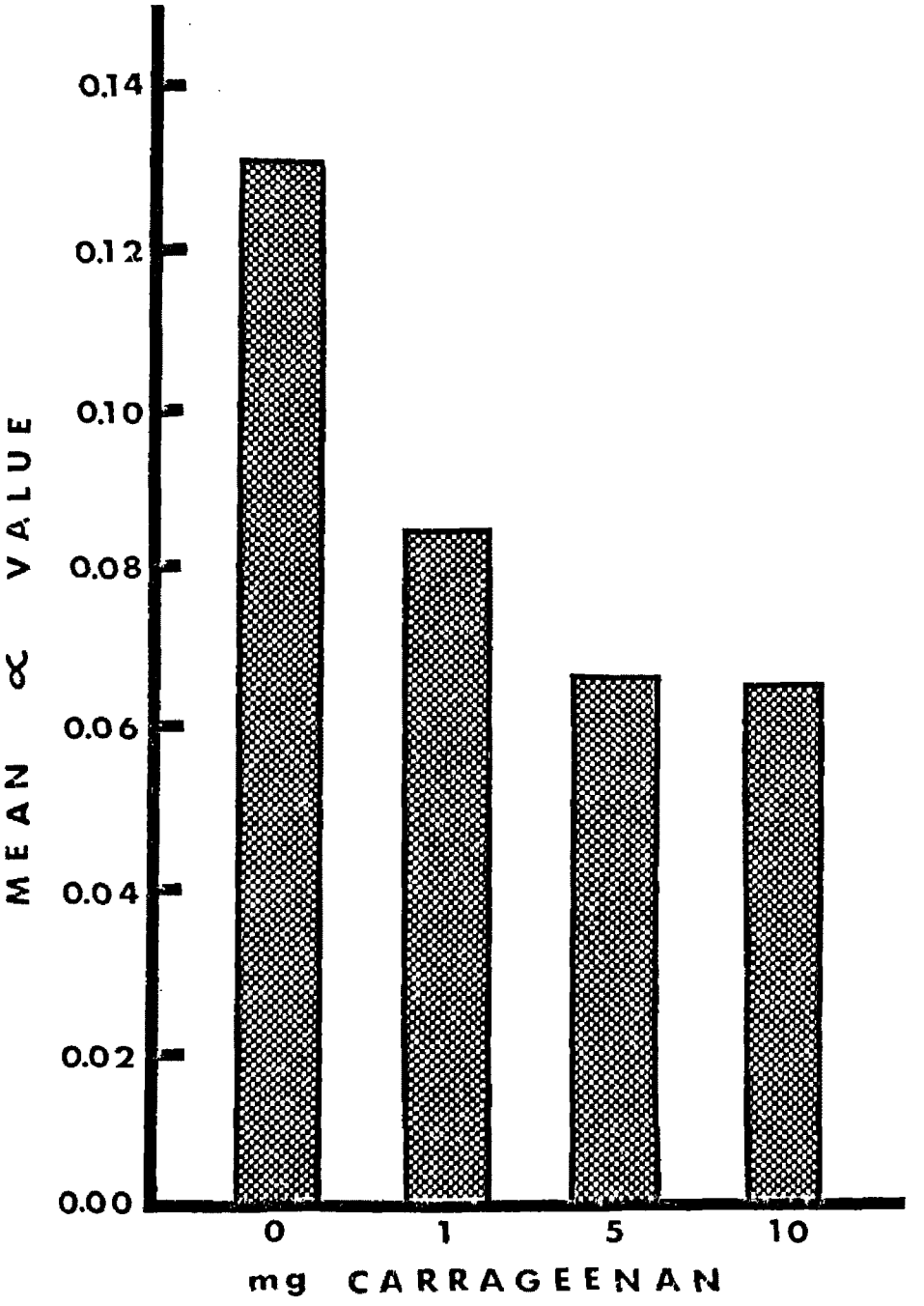
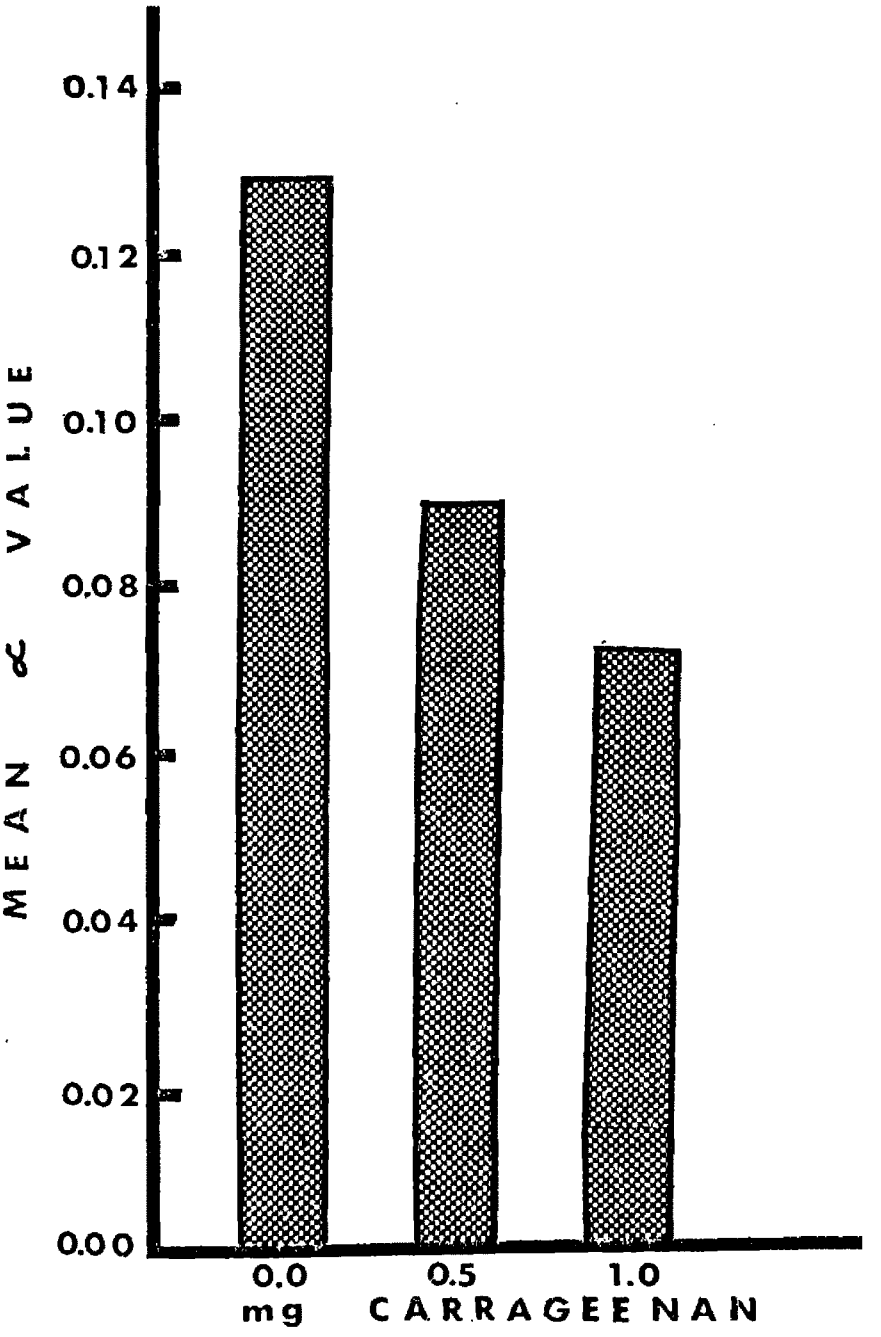


Fig. 4. Phagocytic activity of the RES following i.v. injection of varying amounts of carrageenan. Test mice received a single i.v. injection of carrageenan and were tested for RES activity 24 h later. Normal mice received an equal volume of PBS. Each value represents the arithmetic mean of 5 mice.



100% of the test mice. Therefore, a dose of 5 mg. carrageenan injected i.p. was used in all further experiments; hereafter this will be referred to as the standard dose of carrageenan.

Duration of suppression of RES activity following treatment with MUS

The duration of suppression of RES activity by MUS was evaluated by injecting a group of mice with the standard dose of MUS and subsequently, testing them in groups at varying intervals for their ability to clear colloidal carbon. As shown in Fig. 5, significant ($p < 0.05$) suppression was observed by 2 h after the administration of MUS, and RES activity remained suppressed for 4 days. Maximum suppression was observed at 6 h and continued at this level for at least 24 h. A gradual recovery of RES activity occurred following the single i.v. dose of MUS and clearance values, equivalent to those obtained in normal mice, were obtained by day 5.

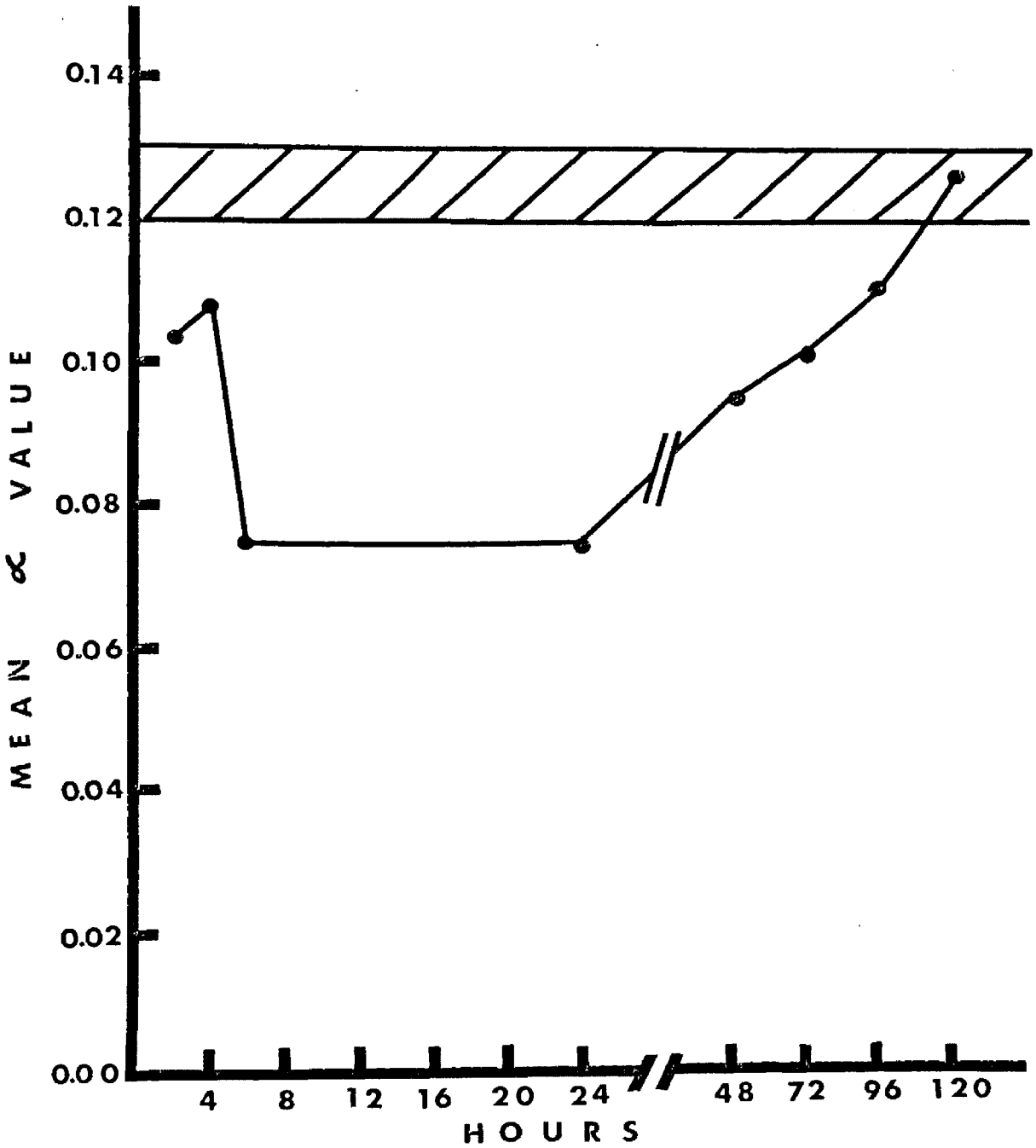
Duration of RES suppression following treatment with carrageenan

The duration of suppression of the RES following carrageenan treatment was evaluated in the same manner as MUS. Mice were injected with the standard dose of

Fig. 5. Duration of RES suppression following treatment with MUS. Test mice received a single i.v. injection of 10 mg MUS at time 0 and were tested at various times for their ability to clear carbon from the peripheral blood. Normal mice received an equal volume of PBS. Each value represents the arithmetic mean of 5 mice.

Symbols:

 Normal range of α value



carrageenan, and then tested at varying times for their ability to clear colloidal carbon from the peripheral blood. Results are shown in Fig. 6. Within 2 h of the carrageenan injection, RES activity was suppressed significantly ($p < 0.05$) and remained suppressed for 4 days. Maximum suppression was observed at 6 h and continued at this level for 48 h.

When Fig. 5 and 6 are compared, the kinetics of suppression obtained after MUS and carrageenan treatment seemed to be similar. Both phagocytic indexes dropped to a minimum mean phagocytic clearance value of 0.07 ± 0.005 within 6 h after treatment. The suppression persisted 24 h with MUS and 48 h with carrageenan, after which a linear recovery occurred and normal phagocytic clearance values were obtained by day 5.

Effects of MUS and carrageenan on phagocytosis in vitro

Since phagocytosis experiments carried out in the whole mouse may reflect a reduction in the phagocytic activity of only the macrophages fixed *in situ*, it was of concern to determine whether wandering macrophages were also impaired. In the following experiment, peritoneal exudate cells were harvested from normal mice and from mice which had been treated with the macrophage toxins. The cells were counted and evaluated for their ability to phagocytize heat-killed *Candida stellatoideae* cells.

Fig. 6. Duration of RES suppression following treatment with carrageenan. Test mice received a single i.p. injection of 5 mg carrageenan at time 0 and were tested at various times for their ability to clear carbon from the peripheral blood. Normal mice received an equal volume of PBS. Each value represents the arithmetic mean of 5 mice.

Symbols:

 Normal range of α value

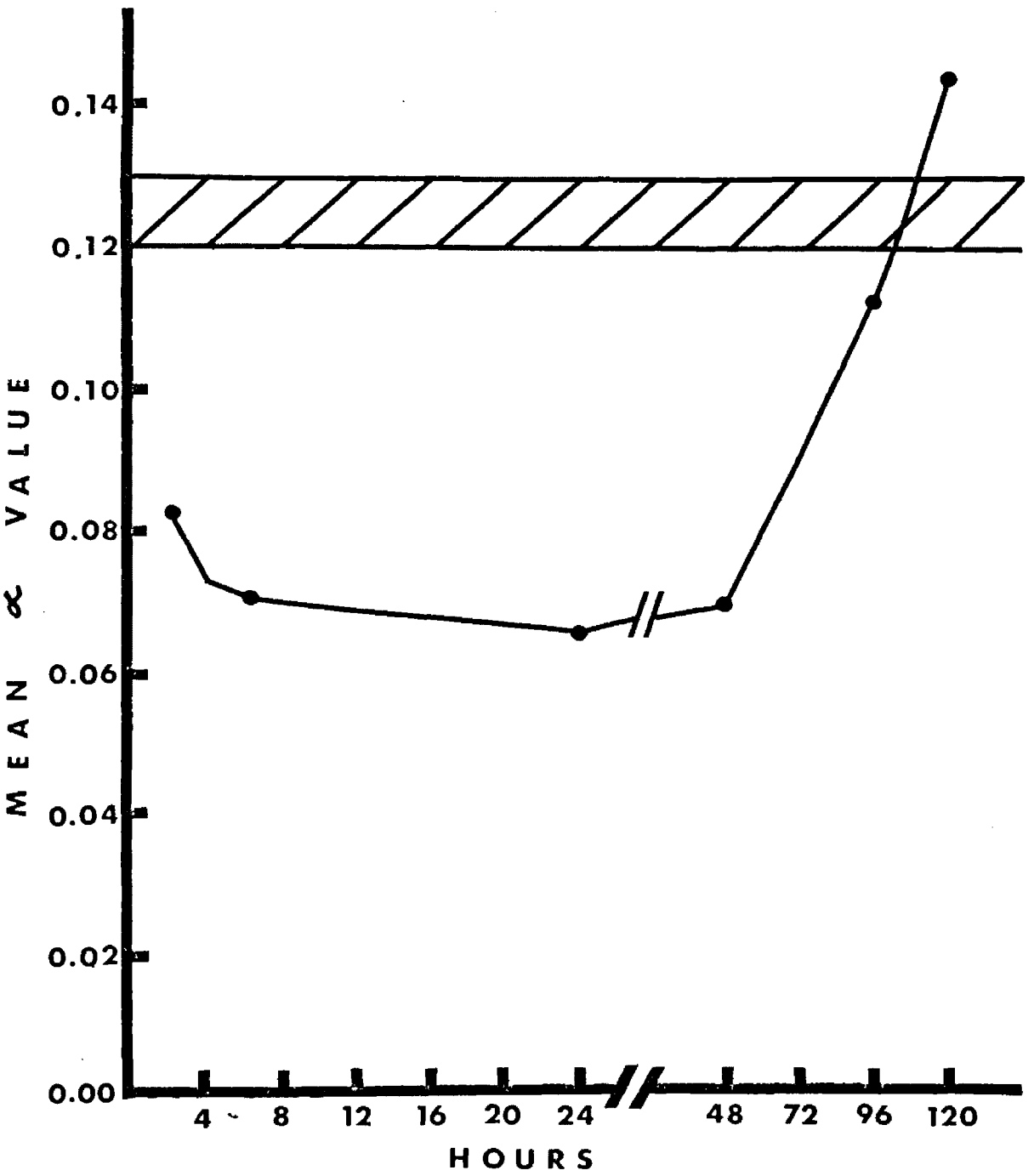


Table I shows that 24 h after MUS or carrageenan treatment, the total number of macrophages harvested from the peritoneal cavity was lower than normal. Concurrent with enumeration, cells were also evaluated for viability by trypan blue exclusion. A lower percent of viable cells were obtained from MUS-treated and carrageenan-treated mice as compared with normal mice. The predominant cell type obtained from normal and MUS-treated mice were lymphocytes, whereas, the predominant cell type in carrageenan-treated mice were polymorphonuclear cells (PMN). When macrophages from MUS-treated and carrageenan-treated mice were incubated with heat-killed C. stellatoideae, the yeast were phagocytized less effectively than by macrophages from control mice which had been injected with only PBS. Thus, not only were fewer viable macrophages obtained from the mice treated with MUS and carrageenan but those that were obtained, had lessened phagocytic activity.

Distribution of radiolabeled LPS in normal and macrophage-impaired mice

The carbon clearance data demonstrated clearly that following the treatment of mice with MUS, there was a suppression in the ability of the RES to clear carbon particles from the peripheral blood. Similarly, the carbon-clearing ability of the RES in carrageenan-treated mice was

Table 1

The Effect of MUS and Carrageenan
on Peritoneal Exudate Cells

Group[a]	Cells/ml	Differential Count (%)			Absolute # Macrophages	Viability[b] (%)	Phagocytic[c] Index
		Lymphocytes	Macrophages	Polymorphs			
Normal[d]	1.2×10^6	55	43	2	5.2×10^5	96	77
MUS[e]	1.3×10^6	63	34	3	4.4×10^5	85	54
Normal[f]	1.3×10^6	57	42	1	5.5×10^5	95	78
Carrageenan[g]	1.7×10^6	6	10	84	1.7×10^5	82	47

[a] 5 mice/group

[b] Trypan Blue exclusion

[c] Percent of adherent cells (macrophages) containing at least 2 Candida stellatoidea.

[d] PBS injected i.v.

[e] 10 mg MUS injected i.v.

[f] PBS injected i.p.

[g] 5 mg carrageenan injected i.p.

impaired. Experiments were performed to determine if the clearance of an antigen was also affected. Therefore, LPS was radiolabeled with ^{51}Cr as described in the Materials and Methods section. The radioactive LPS (LPS*) was injected i.v. into mice previously treated with the standard dose of MUS or carrageenan. Control mice received PBS by identical routes.

The ability to clear the LPS* by mice exposed to either macrophage toxin was dramatically impaired (Table 2 and 3). By 5 min following the i.v. injection of LPS* into normal mice, only about 4% of the total injected concentration could be recovered in whole blood. However, in mice treated previously with MUS prior to the i.v. injection of LPS*, about 40% of the antigen injected was present in whole blood. Similarly, carrageenan-treated mice retained about 43% of the injected LPS* in the circulating whole blood after 5 min. The concentration of LPS* within the circulating whole blood in all groups diminished with time. However, the rate of LPS* clearance from the blood in mice exposed to MUS or carrageenan was less than the clearance rate in normal mice.

As seen in Table 2, LPS* was quantified also in various organs. Prior to counting, the individual organs were rinsed with PBS but were not perfused. Therefore, the counts in the organs obtained while the peripheral blood

Table 2

Tissue Distribution of ^{51}Cr-labeled LPS in Normal and Macrophage-impaired Mice

Group[a]	Organ	Nanograms of LPS[b]					
		Time of Assay					
		5 min.	30 min.	1h	6h	24h	48h
Normal	Blood[c]	364	272	108	84	48	28
	Spleen	78	56	41	42	54	46
	Liver	7837	7709	7690	7406	7434	7340
	Kidneys	65	45	36	37	31	32
	Lungs	27	29	9	7	13	7
MUS[d]	Blood[c]	4068	3276	2880	1824	544	248
	Spleen	973	836	778	787	887	864
	Liver	1644	1568	2180	2424	3196	3290
	Kidneys	207	216	238	189	118	119
	Lungs	174	318	77	44	75	63
Carrageenan[e]	Blood[c]	4324	3900	4164	ND[f]	ND	ND
	Spleen	223	206	204	ND	ND	ND
	Liver	1278	1849	1760	ND	ND	ND
	Kidneys	186	206	296	ND	ND	ND
	Lungs	190	273	220	ND	ND	ND

[a] All mice received 10,000 ng of ^{51}Cr-labeled LPS i.v. at time 0.

[b] Nanograms of LPS were calculated by the following formula:
ng of LPS = (10,000 ng LPS) (counts/organ) / [100,394 counts/10,000 ng LPS]
All samples were counted for the same period of time and were corrected for background and decay. Each value represents the arithmetic mean of 3 mice/group.

[c] 0.5 ml of blood was counted. Amount of LPS was calculated assuming a circulating blood volume of 2.0 ml

[d] Mice received 10 mg MUS i.v. at time -6 h.

[e] Mice received 5 mg carrageenan i.p. at time -6 h.

[f] N.D. Not done

Table 3

Percent Tissue Distribution of ^{51}Cr-labeled LPS in Normal and Macrophage-impaired Mice

Group[a]	Organ	Percent of Total Injected LPS[b]					
		Time of Assay					
		5 min.	30 min.	1 h	6 h	24 h	48 h
Normal	Blood[c]	3.6	2.7	1.1	0.8	0.5	0.3
	Spleen	0.8	0.6	0.4	0.4	0.5	0.5
	Liver	78.4	77.1	76.9	74.1	74.3	73.4
	Kidneys	0.7	0.5	0.4	0.4	0.3	0.3
	Lungs	0.3	0.3	0.1	0.1	0.1	0.1
MUS[d]	Blood[c]	40.7	32.8	28.9	18.2	5.4	2.5
	Spleen	9.7	8.4	7.8	7.9	8.9	8.6
	Liver	16.4	15.7	21.8	24.2	32.0	32.9
	Kidneys	2.1	2.2	2.4	1.9	1.2	1.2
	Lungs	1.7	3.2	0.8	0.4	0.8	0.6
Carrageenan[e]	Blood[c]	43.2	39.0	41.6	ND[f]	ND	ND
	Spleen	2.3	2.1	2.0	ND	ND	ND
	Liver	12.8	18.5	17.6	ND	ND	ND
	Kidneys	1.9	2.1	3.0	ND	ND	ND
	Lungs	1.9	2.7	2.2	ND	ND	ND

[a] All mice received 10,000 ng of ^{51}Cr-labeled LPS i.v. at time 0.

[b] Percent of total injected LPS = $\frac{\text{ng of LPS in organ}}{\text{ng of LPS injected}} \times 100$

[c] 0.5 ml of blood was counted. Percent of LPS was calculated assuming a circulating blood volume of 2.0 ml.

[d] Mice received 10 mg MUS i.v. at time - 6 h.

[e] Mice received 5 mg carrageenan i.p. at time - 6 h.

[f] N.D. Not done.

LPS* concentration was high, probably reflected some LPS* contained in the blood in addition to the specific localization of LPS* within these various organs. It can be seen that a relatively small amount of the total LPS* injected was found in the kidneys and lungs. However, the liver and spleen showed considerable localization of LPS*. In normal control mice 78% and 0.8% of the total injected LPS* were found in the liver and spleen respectively, after 5 min. MUS-treated mice revealed 16% LPS* recovered from the liver while 10% was recovered from the spleen. It was probable that the increased amount of LPS* seen within the spleen in MUS-treated mice represented an increased localization of LPS* and not merely entrapment of peripheral blood, since this value remained constant throughout the experiment and did not decrease proportionally with the amount of LPS* in the peripheral blood. Similarly, in carrageenan-treated mice, 13% and 2% of the injected LPS* was recovered from the liver and spleen, respectively, after 5 min. Again it was assumed that the increase in splenic LPS* represented a true increased localization of LPS*, since the increased values persisted at this level throughout the duration of the experiment.

Localization of LPS* within the liver of treated mice increased with time throughout the experiment. After 48 h the amount of LPS* recovered from the liver of MUS-treated mice had increased to 33% of that injected, while in

carrageenan-treated mice approximately 20% of the injected LPS* was localized in the liver. By comparison, normal mice retained 75% of the injected LPS* at 48 h.

Thus, additional evidence was obtained which showed that MUS and carrageenan had impaired macrophages. The increase in splenic localization of LPS* was relatively greater than in other tissues in treated mice. Organs which were examined for localization of LPS* included stomach, intestines, bladder, mesenteric lymph nodes, inguinal lymph nodes, axillary lymph nodes and popliteal nodes. However, none of these appeared to be specific sites for localization of LPS* in either normal or treated mice. These data may be important in light of further observations presented in the next sessions.

Sensitivity of MUS and carrageenan-treated mice to LPS toxicity

It is well established that agents which either blockade or reduce RES function render animals more susceptible to the toxicity of LPS (37, 93). Based on this observation, the median lethal dose (LD_{50}) to LPS should reflect the *in vivo* functional capacity of the RES. Mice were injected with 10 mg MUS i.v. or with 5 mg carrageenan i.p. These injections were followed by the i.p. injection of varying amounts of LPS. Controls consisted of animals receiving only MUS or carrageenan but no LPS and normal mice

receiving only the LPS. The mice were observed and deaths recorded at 12 h intervals until termination of the experiment at 72 h.

Figure 7 shows that mice receiving LPS concurrently with either MUS or carrageenan were more susceptible to the toxicity of LPS than were normal mice. MUS-treated mice were approximately 40 times more sensitive to LPS toxicity than normal mice. An LD_{50} value of 2.7×10^4 ng of LPS (Fig. 8) was obtained with MUS-treated mice, whereas normal mice exhibited no deaths even at the highest dose of LPS (1×10^6 ng). Carrageenan-treated mice were approximately 3,000 times more sensitive to LPS toxicity with an LD_{50} value of 3.7×10^2 ng of LPS. Hence, the LD_{50} values obtained with MUS-treated and carrageenan-treated mice provided additional evidence that impairment of the RES had occurred.

Primary antibody responses of mice given antigen at varying times during macrophage impairment

Other authors have presented data indicating that the time at which mice were challenged with antigen during the course of macrophage impairment was critical to the resultant antibody response (52, 72, 81). Therefore, experiments were designed to expose mice to antigen at varying intervals prior to and after the dose of macrophage toxins.

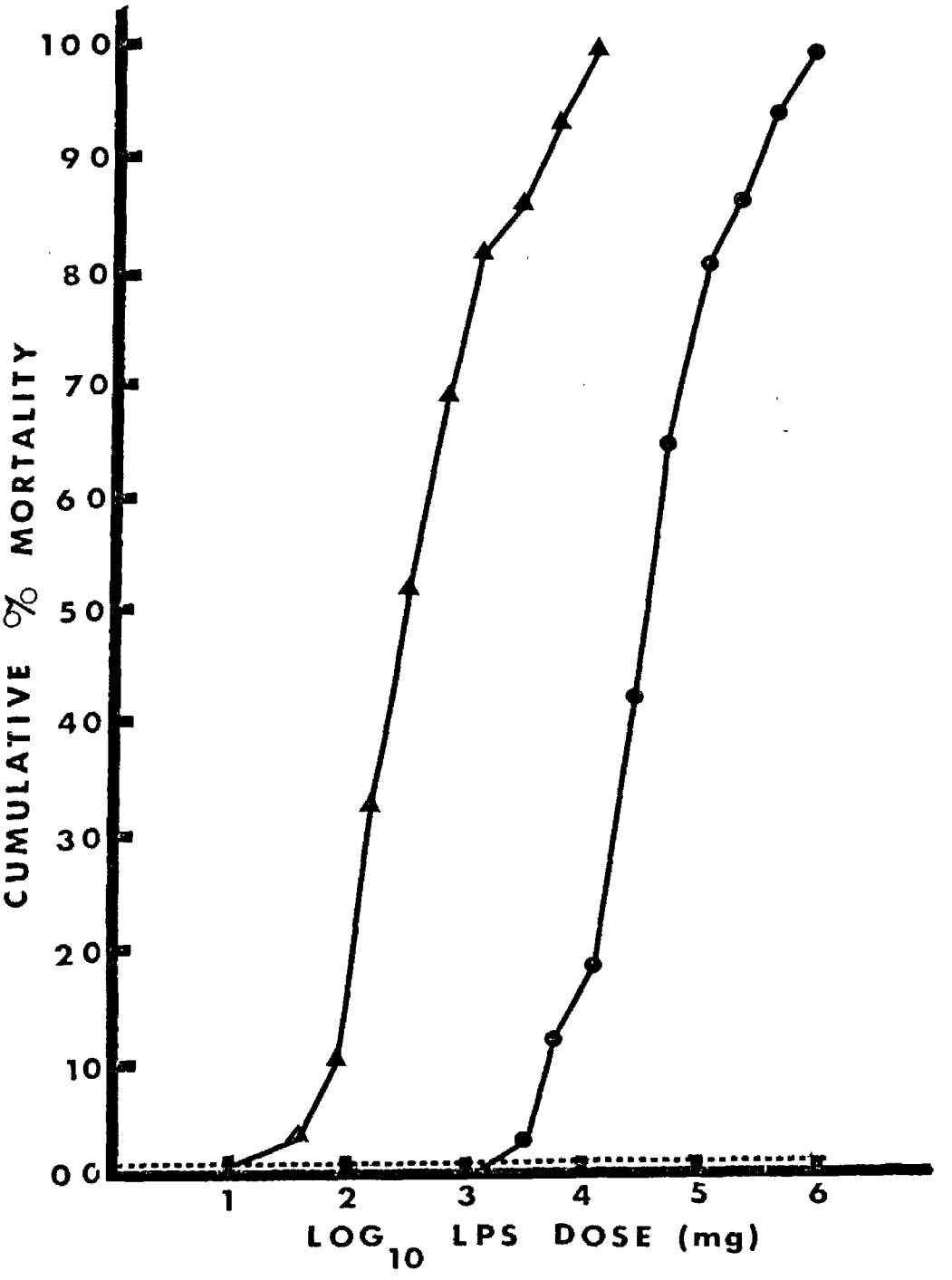
Fig. 7. Sensitivity of MUS or carrageenan-treated mice to LPS toxicity. Mice were injected with 10 mg of MUS i.v. or 5 mg of carrageenan i.p. at time 0, followed by the injection of varying amounts of LPS i.p. Normal mice received PBS by identical routes followed by the injection of LPS i.p.

Symbols:

Normal mice


Carrageenan-treated mice


MUS-treated mice



Mice were challenged with antigen as early as 24 h prior to the standard dose of MUS (carrageenan) or as late as 5 days after the injection of macrophage toxin. Mice were bled 5 days after the dose of antigen and their sera were pooled and titrated for antibody.

As shown on Table 4, if either LPS or SRBC antigen was given prior to or concurrently with either macrophage toxin there was no observable difference in the resultant antibody titer as compared to normal mice. The only observable difference in titer between normal mice and mice injected with macrophage toxins occurred when mice were challenged with 0.2 ml of 0.05% SRBC suspension 6 h and 24 h after exposure to either macrophage toxin. As seen on Table 4, mice that received MUS or carrageenan demonstrated an enhanced antibody titer to the lower dose of SRBC. Mice that received antigen later than 24 h after exposure to macrophage toxins did not demonstrate an observable difference in the antibody response.

It was found in this experiment that the only observable difference in the antibody response occurred when mice received a sub-maximal SRBC antigenic challenge 6 h and 24 h after exposure to MUS or carrageenan. However, at no time during RES suppression by either macrophage toxin was there a decrease in antibody response, regardless of the time of exposure to the toxin.

Table 4

Primary Antibody Responses of Mice Immunized at Varying Intervals During Macrophage Impairment

Group[a]	Antigen	Antibody Titer[b]								
		Time of Antigen Administration (hours)								
		-24	-6	0	6	24	48	72	96	120
PBS	10 ug LPS	7	8	8	7	7	8	7	7	8
	0.1 ug LPS	5	5	6	4	5	5	4	5	5
	10% SRBC	7.5	8	7	8	7.5	8	8	7	7.5
	0.05% SRBC	4	4.5	5	4	4	4	4.5	4	5
MUS	10 ug LPS	7	8	8	7	6	7	7	6	6
	10% SRBC	7	8	8	7	7	7	7	8	7
	0.05% SRBC	5	5	5	6	6	4.5	5	5	4
Carrageenan	0.1 ug LPS	6	4	5	5	5	4	6	4	6
	10% SRBC	7	7	8	7.5	7	7	8	8	7
	0.05% SRBC	4.5	4	4	6	6	4	3.5	4	4.5

[a]PBS, 10 mg MUS or 5 mg carrageenan injected at time 0 (5 mice/group).

[b]Mice were bled 5 days after antigenic challenge, sera pooled and titers determined.

Kinetics of the primary antibody response to LPS and SRBC in MUS-treated mice

The kinetics of the primary antibody response to LPS was evaluated in MUS-treated and normal mice. Animals received the standard dose of MUS and 6 h later were challenged i.v. with antigen. Control groups received an equal volume of PBS i.v. and were challenged similarly with antigen. Groups of 5 mice were bled daily, their sera pooled and evaluated for antibodies to LPS by passive hemagglutination. As shown in Fig. 8, there does not appear to be a difference in either the kinetics or in the magnitude of the resulting antibody response. With either dose of LPS (10 ug or 1 ug), initial antibody responses could be detected after 3 days. Maximum antibody titers in treated mice, as well as normal mice were obtained on day 5 and persisted at this level for the duration of the experiment.

Sera obtained on the fifth day were also treated with 2-mercaptoethanol (2-ME) in order to estimate the relative amounts of antibody in the IgM and IgG classes. Serum samples were treated with 2-ME, as described in the Materials and Methods section, and then titrated for the presence of specific antibody activity. When the titers of the sera before and after treatment with 2-ME were compared it was possible to determine the relative contribution if IgM and IgG antibodies to the total response. Following

Fig. 8. Kinetics of the primary antibody response of normal and MUS-treated mice to LPS. Mice were injected at time 0 with 10 mg MUS or PBS i.v. followed 6 h later with the injection of LPS. Mice were bled on 8 consecutive days and titers were determined by passive hemagglutination. Each point represents the antibody titer of sera pooled from 5 mice/group.

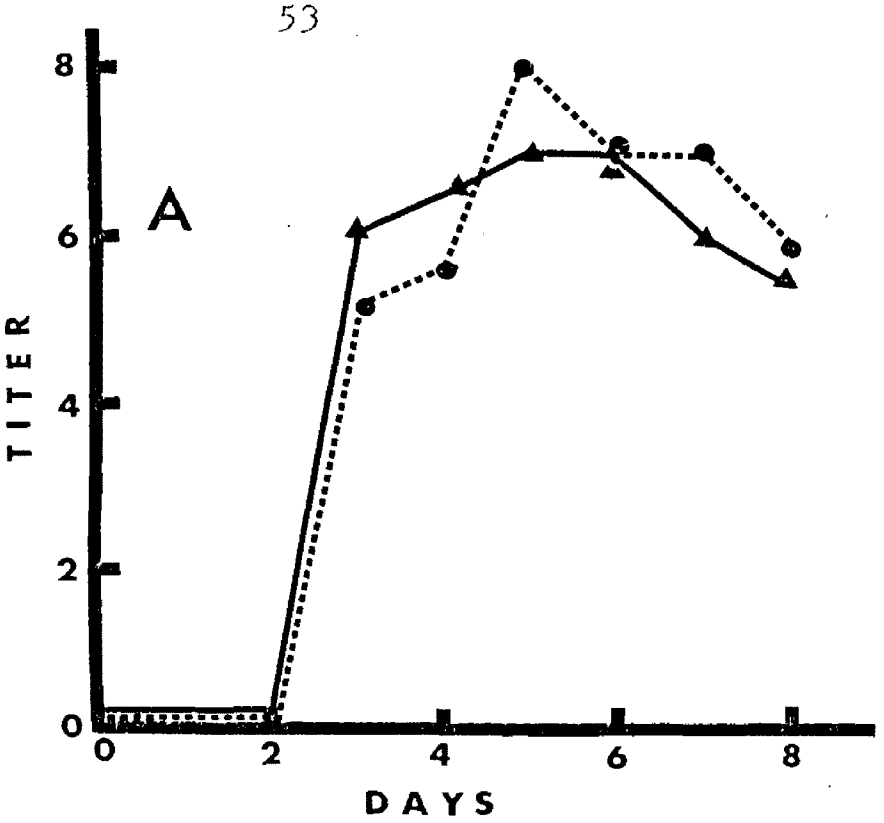
Symbols:

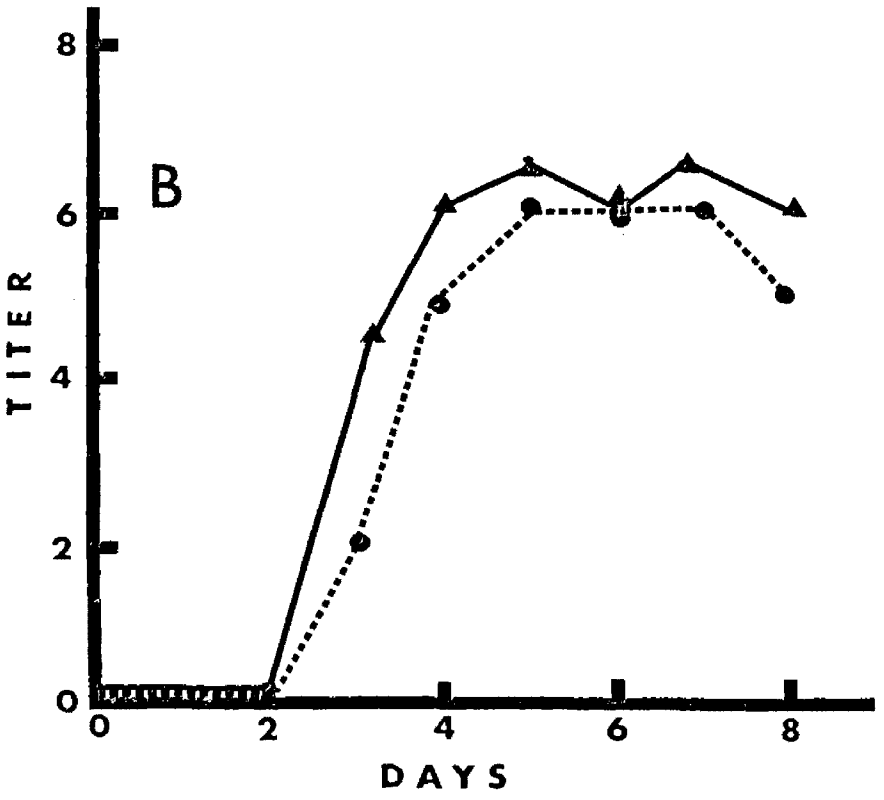
—▲——▲— Normal mice

·●·····●· MUS-treated mice

A. Mice injected with 10 ug LPS, i.v.

B. Mice injected with 1 ug LPS, i.v.





treatment with 2-ME there was no residual antibody titer detectable by passive hemagglutination. Therefore, the antibody response for both groups of mice was primarily IgM.

Treated and normal mice were also evaluated for their ability to respond to the T-cell dependent SRBC antigen. The experimental protocol was the same as that described previously for LPS, except that SRBC was used as the antigen. With the high antigenic dose of SRBC (0.2 ml of 10% SRBC) initial antibody responses could be detected 2 days after antigenic stimulation in both groups of mice. (Fig. 9 A). After the second day the kinetics of the antibody response were similar for both groups until approximately the seventh day. At that time the MUS-treated mice attained a peak titer of 11, while the normal mice peaked at a titer of 8.5. When mice were challenged with the submaximal dose of SRBC (0.2 ml of 0.05% SRBC) both the kinetics and magnitude of the antibody response varied. Circulating antibodies to SRBC could be detected in macrophage-impaired mice at 2 days, but were not detectable in normal mice until 4 days after antigenic stimulation (Fig. 9 B). Not only were circulating antibodies demonstrable earlier in macrophage-impaired mice, but the maximum antibody response was 4 fold (end point dilution of 192 vs 48) higher than the maximum antibody response generated by normal mice.

Fig. 9. Kinetics of the primary antibody response of normal and MUS-treated mice to SRBC. Mice were injected at time 0 with 10 mg MUS or PBS i.v. followed 6 h later by the injection of SRBC. Mice were bled on 8 consecutive days and titers were determined by hemagglutination. Each point represents the antibody titer of sera pooled from 5 mice/group.

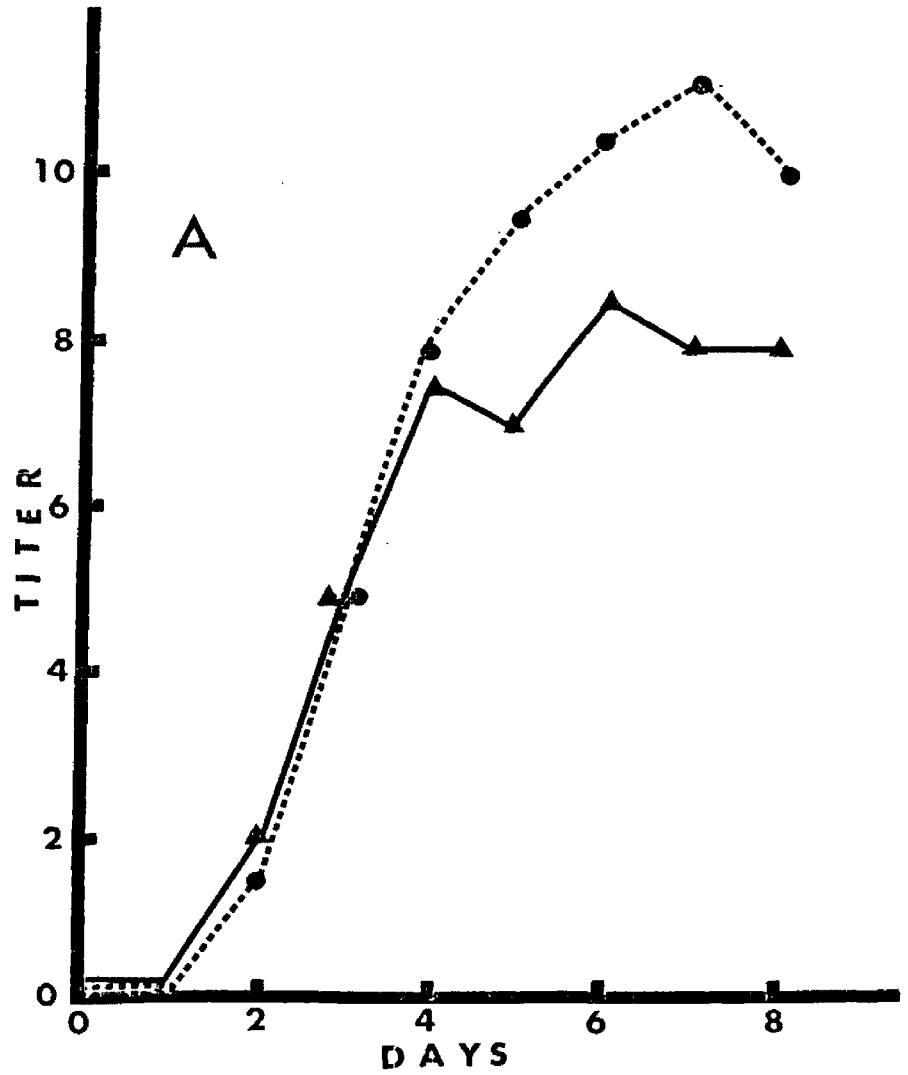
Symbols:

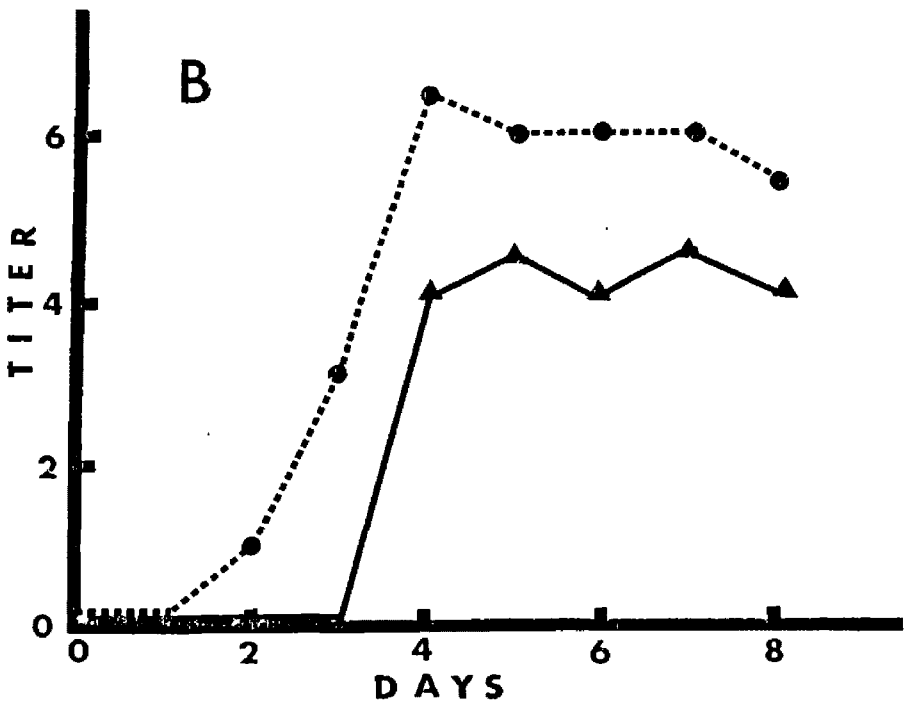
 Normal mice

 MUS-treated mice

A. Mice injected with 0.2 ml of 10% SRBC, i.v.

B. Mice injected with 0.2 ml of 0.05% SRBC, i.v.





Kinetics of the primary antibody response to LPS and SRBC in carrageenan-treated mice

The kinetics of the primary antibody response to LPS was evaluated in carrageenan-treated and in normal mice. Mice were injected with the standard dose of carrageenan or PBS and 6 h later were injected i.v. with antigen. Because of the increased susceptibility to LPS in the carrageenan-treated mice, only one dose of LPS (0.1 ug) was used. As shown in Fig. 10, there was not any difference in either the kinetics or in the magnitude of the antibody response between macrophage-impaired and normal mice. Initial antibody responses could be detected in both groups, 3 days after the injection of antigen. Peak titers for both groups were reached on day 5 and titers tended to persist at this level for the duration of the experiment.

The primary antibody response of carrageenan-treated mice to SRBC was evaluated in a manner similar to that described above. Mice received the standard dose of carrageenan or PBS followed 6 h later by the i.v. injection of SRBC. With the larger dose of SRBC (0.2 ml of 10% SRBC), both the time of development and magnitude of the resulting antibody titers were similar in both carrageenan-treated and normal mice (Fig. 11 A). Initial antibody responses were demonstrable 2 days after antigenic challenge. Peak titers in both groups were achieved 5 days after antigenic

Fig. 10. Kinetics of the primary antibody response of normal and carrageenan-treated mice to LPS. Mice were injected at time 0 with PBS or 5 mg carrageenan i.p. followed 6 h later by the injection of LPS. Mice were bled on 8 consecutive days and titers were determined by passive hemagglutination. Each point represents the antibody titer of sera pooled from 5 mice/group.

Symbols:

—▲——▲— Normal mice, injected i.v. with 0.1 ug of LPS

··●·····●·· Carrageenan-treated mice, injected with 0.1 ug of LPS

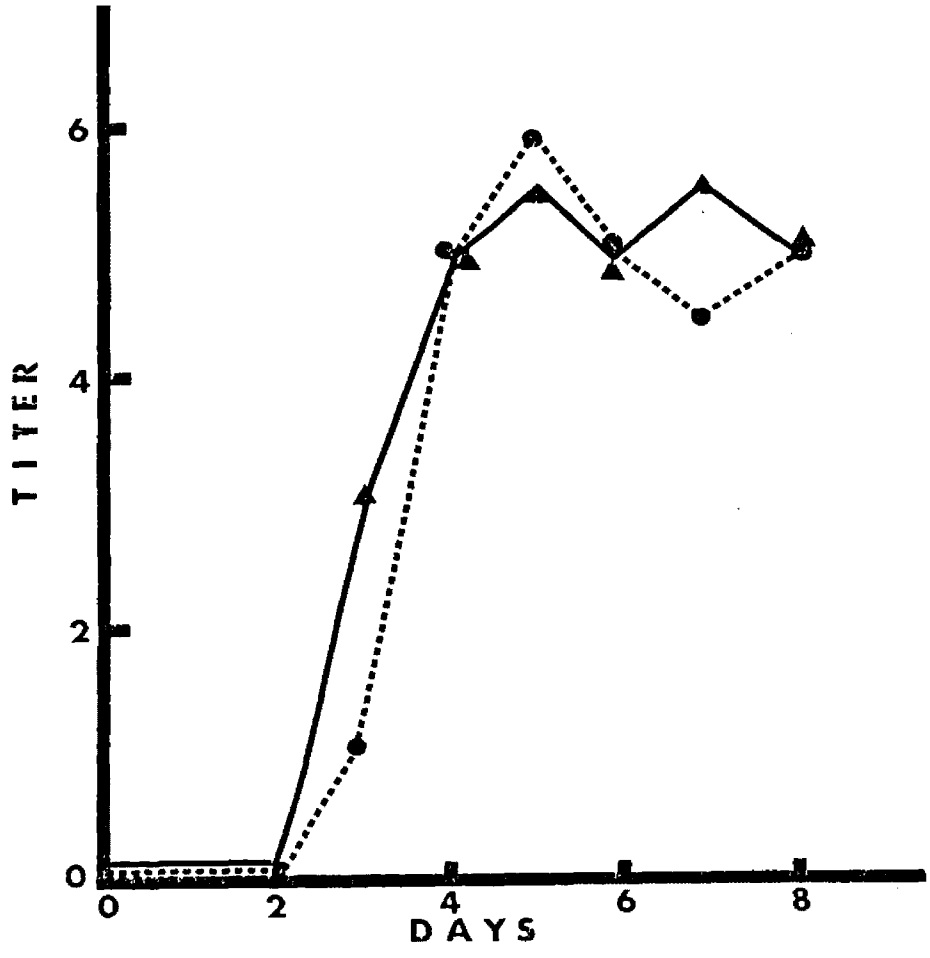


Fig. 11. Kinetics of the primary antibody response of normal and carrageenan-treated mice to SRBC. Mice were injected at time 0 with PBS or 5 mg carrageenan i.p. followed 6 h later by the injection of SRBC. Mice were bled on 8 consecutive days and titers were determined by hemagglutination. Each point represents the antibody titer of sera pooled from 5 mice/group.

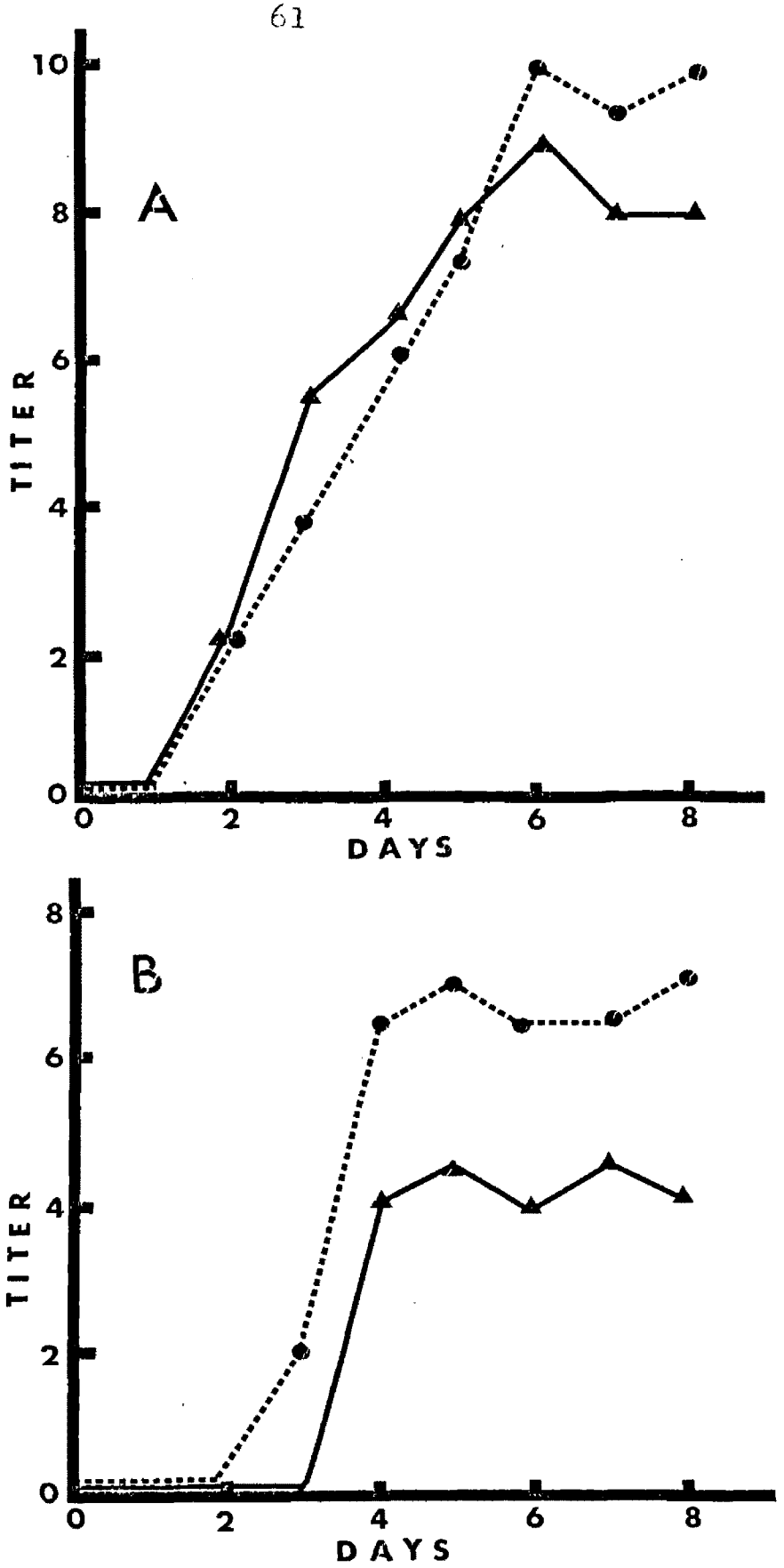
Symbols:

—▲——▲— Normal mice

--●·····●-- Carrageenan-treated mice

A. Mice injected i.v. with 0.2 ml of 10% SRBC.

B. Mice injected i.v. with 0.2 ml of 0.05% SRBC.



challenge and tended to persist at this level for the duration of the experiment. When mice received the sub-maximal dose of SRBC (0.2 ml of 0.05% SRBC) both the kinetics and the magnitude of the antibody response were altered (Fig. 11 B). Initial antibody responses were detectable 1 day earlier in carrageenan-treated mice than in normal mice. Maximum anti-SRBC titers were obtained 5 days after the injection of antigen in both groups, however, there was an approximate 5 fold (end point dilution of 256 vs 48) increase in the magnitude of the antibody response of the carrageenan-treated mice.

Primary antibody responses of normal and macrophage-impaired mice to various concentrations of SRBC

During the characterization of the kinetics of the primary antibody response to the SRBC antigen, it was observed that MUS-treated or carrageenan-treated mice demonstrated higher antibody titers in their sera than normal mice. Further experiments were performed to determine if the enhanced primary antibody response to SRBC was dose dependent.

Groups of 5 mice were treated with the standard dose of MUS or carrageenan. Control groups received an equal volume of PBS by identical routes of injection. After 6 h, mice were injected with varying amounts of SRBC. Five days after the dose of antigen, the mice were bled, their sera

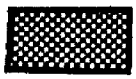
pooled and titrated.

Figures 12 and 13 demonstrate that as the dose of SRBC was decreased, the resulting antibody titer decreased but still remained at an enhanced level in the MUS-treated and carrageenan-treated mice when compared to normal mice. In contrast, normal mice exhibited a more profound drop in the resultant antibody titer as the dose of antigen was decreased. Anti-SRBC titers of 7.5, 6 and 4 were obtained with 0.2 ml of 10%, 1% and 0.05% SRBC, respectively, in normal mice. MUS-treated mice demonstrated anti-SRBC titers of 9.5, 8 and 7 to similar doses of antigen. Antibody titers of 7, 7 and 6.5 were obtained respectively in carrageenan-treated mice. Thus, the primary antibody response to the SRBC antigen were consistently higher in mice previously treated with MUS or carrageenan.

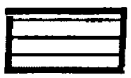
The sera from both normal and macrophage-impaired mice were treated with 2-ME. As shown in Figs. 12 and 13, all mice receiving the primary antigenic challenge dose of 0.2 ml of 10% SRBC, possessed 2-ME resistant antibody activity. Normal mice exhibited a 12 fold (end point dilution of 384 vs 32) reduction in hemagglutinating activity following treatment of the sera with 2-ME, whereas MUS-treated mice showed only a 6 fold (end point dilution of 1536 vs 256) reduction in hemagglutinating activity. Similarly, carrageenan-treated mice showed an 8 fold (end

Fig. 12. Primary antibody responses of normal and MUS treated mice to varying doses of SRBC. Mice were injected at time 0 with PBS or 10 mg of MUS i.v. followed 6 h later with the i.v. injection of SRBC. Mice were bled 5 days later and titers were determined by hemagglutination. Each value represents the antibody titer of sera pooled from 5 mice/group.

Symbols:



Antibody titer of sera



Antibody titer of sera following treatment with 2-mercaptoethanol

A. Normal mice

B. MUS-treated mice

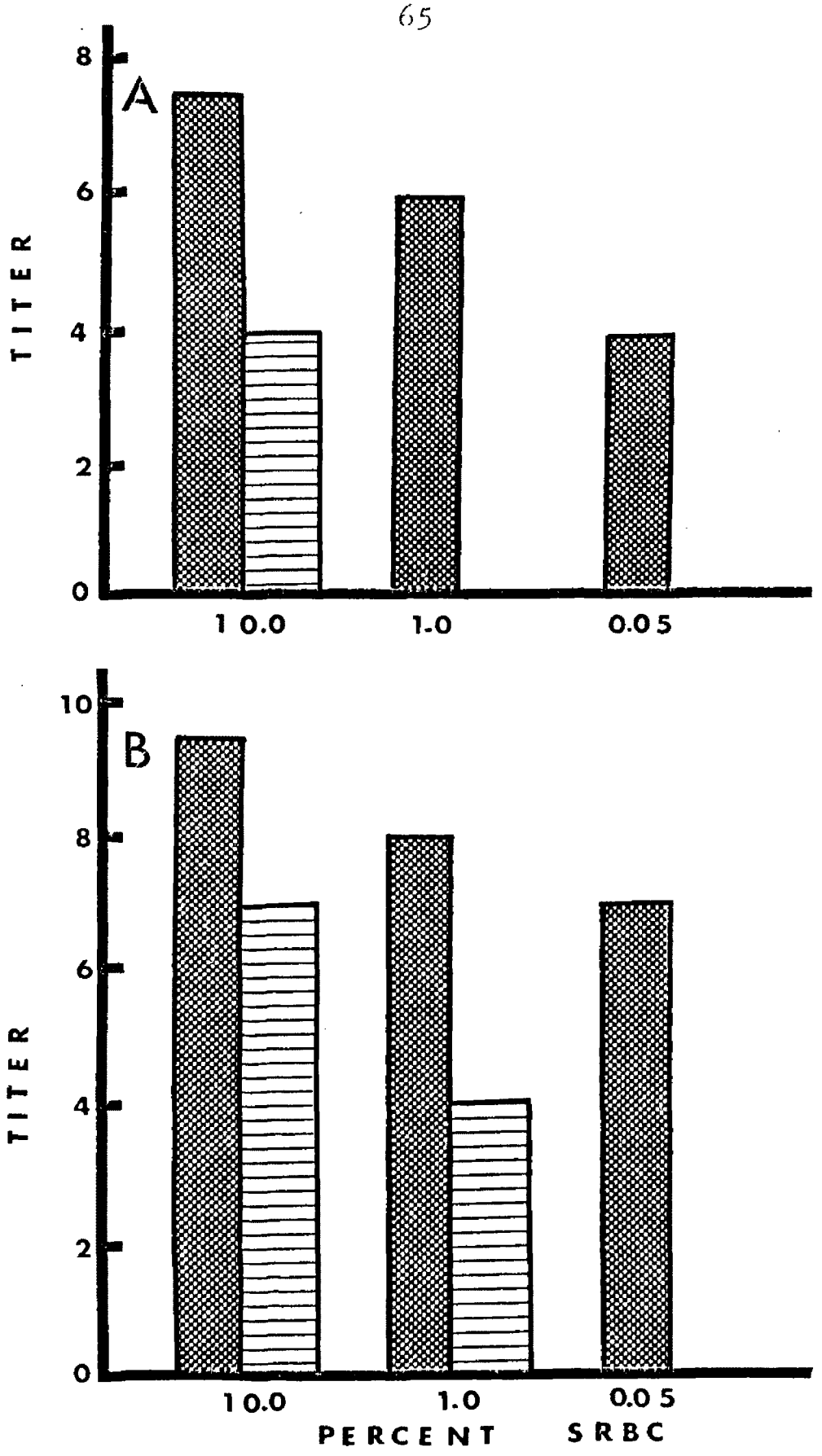
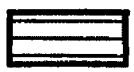


Fig. 13. Primary antibody response of normal and carrageenan-treated mice to varying doses of SRBC. Mice were injected at time 0 with PBS or 5 mg of carrageenan i.p. followed 6 h later with the i.v. injection of SRBC. Mice were bled 5 days later and titers were determined by hemagglutination. Each value represents the antibody titer of sera pooled from 5 mice/group.

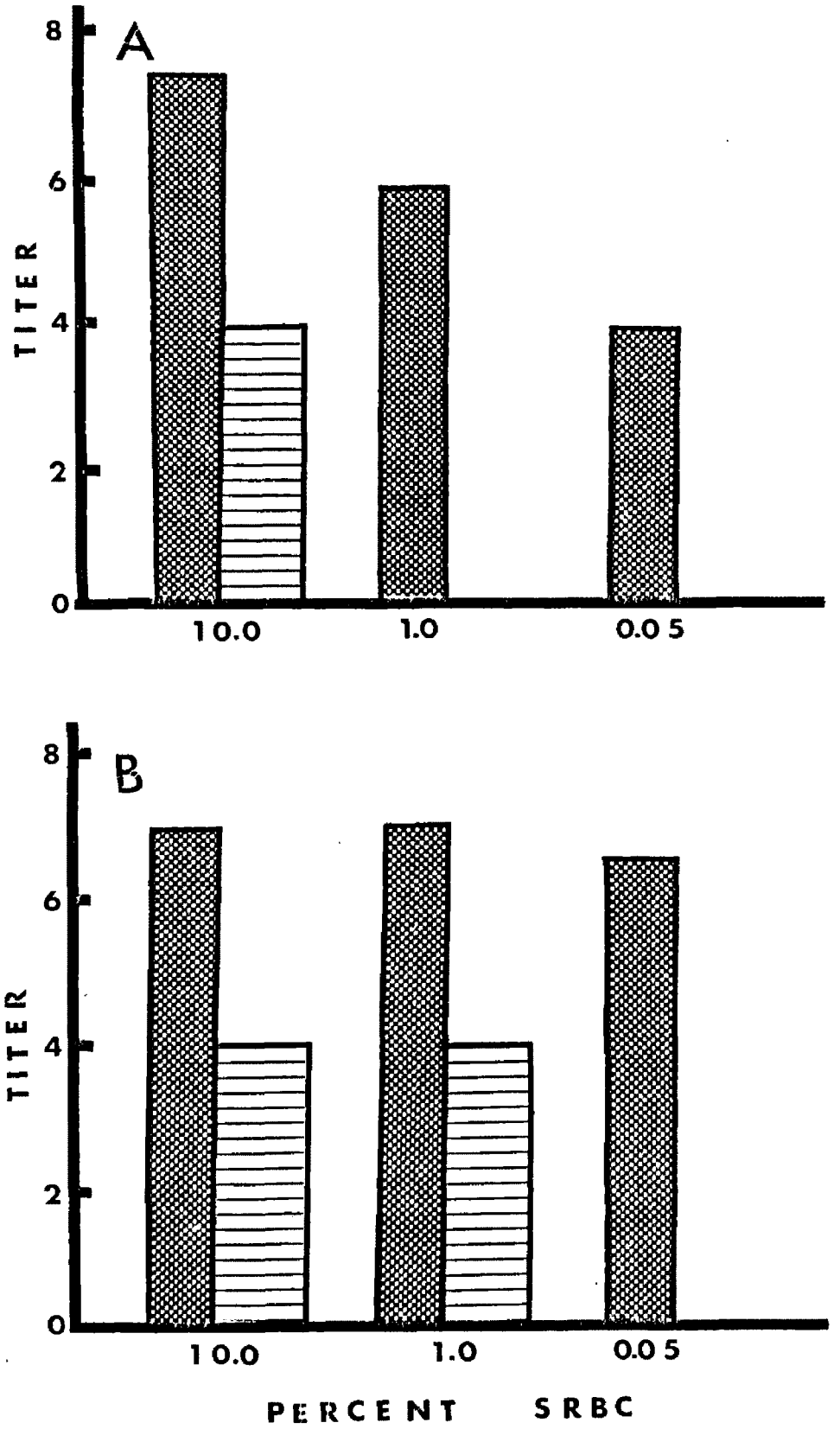
Symbols:

 Antibody titer of sera

 Antibody titer of sera following treatment with 2-mercaptoethanol

A. Normal mice

B. Carrageenan-treated mice



point dilution of 256 vs 32) reduction in titer following treatment of sera with 2-ME. Thus, the sera of mice immunized during macrophage impairment contained more IgG than did the sera of normal mice.

MUS or carrageenan-treated mice were also capable of generating a 2-ME resistant antibody response to an antigenic challenge dose of 0.2 ml of 1% SRBC. Mice immunized during macrophage impairment with MUS or carrageenan demonstrated a 16 to 8 fold (end point dilution of 512 vs 32 and 256 vs 32) reduction in the total antibody titer following 2-ME treatment, respectively. Conversely, in normal mice immunized with this dose of SRBC no 2-ME resistant antibodies were found. When the antigen concentration was reduced to a 0.05% SRBC suspension no 2-ME resistant activity was present in either the macrophage impaired or control group.

Thus, when the higher dose of SRBC (0.2 ml of 10% SRBC) was employed, the resulting antibody response in both normal and treated animals was a mixture of IgG and IgM antibodies. Conversely, with a lower dose of SRBC (0.2 ml of 0.05%) only IgM antibodies were generated in both normal and treated animals. At the intermediate SRBC dose, specific antibodies of IgM and IgG classes were generated only in the mice exposed to macrophage toxins prior to antigenic challenge, while normal control mice responded

with antibody production in only the IgM class.

Release of Hemoglobin following exposure of SRBC to MUS or carrageenan

Feldmann and co-workers (27, 28) had shown previously that the production of an antibody response to SRBC *in vitro* was dependent upon three cell types: T-lymphocytes, B-lymphocytes and macrophages. They were also able to demonstrate, with *in vitro* experiments, that macrophages were required only when intact SRBC were used as antigens. If the SRBC were sonicated and presented to only the T cells and B cells in the soluble form, it was noted that antibody production would occur without the presence of macrophages. Thus, it appeared that the soluble form of the SRBC antigen was macrophage independent *in vitro*, whereas the intact SRBC antigen was macrophage dependent.

Since primary antibody responses to SRBC in macrophage-impaired mice were equal to or greater than the antibody response of normal mice, it was of importance to determine if this enhancement possibly was due to a lytic action of MUS or carrageenan on the SRBC *in vivo*. If this was occurring the role of the macrophage as a possible antigen-processing cell would be circumvented. Therefore, MUS and carrageenan were analyzed *in vitro* for their ability to lyse SRBC.

As shown in Table 5, there was no difference in the free hemoglobin levels between control sera and sera plus *in vivo* concentrations of MUS or carrageenan. If either macrophage toxin were capable of inducing lysis of the SRBC, this should have been reflected in a higher free hemoglobin level. Since this was not observed with either MUS or carrageenan, it could be surmised that the *in vivo* amounts of macrophage toxins employed in the present study were not lytic to SRBC.

Passive transfer of sera from normal and macrophage-impaired mice into normal mice which received LPS or SRBC

The destruction of macrophages *in vivo* by MUS or carrageenan should result in the release of intracellular enzymes into the peripheral blood. Measurement of these enzymes would offer a method for determining the extent of macrophage destruction that had occurred. Therefore, experiments were designed to assay the sera from mice receiving MUS or carrageenan for acid phosphatase and lactate dehydrogenase (LDH) levels. These same sera were then transferred passively into normal mice, which subsequently were challenged with antigen and their antibody titers determined.

Mice were treated with the standard dose of MUS or carrageenan. Control mice received an equal volume of PBS by identical routes of injection. The mice were

Table 5

Release of Hemoglobin Following Exposure of SRBC to MUS or Carrageenan

Group	Incubation Mixture			Free Hemoglobin(mg/dl)
	A	B	C	
Controls	serum	serum	serum	12
	serum	serum	SRBC	12
	serum	PBS	SRBC	12
	serum	water	SRBC	20
	water	water	SRBC	520
MUS	serum	MUS	SRBC	11
Carra-geenan	serum	Carra-geenan	SRBC	12

A. 1 ml of serum or water.

B. 0.2 ml of serum, PBS, water, MUS or carrageenan.

C. 0.2 ml of serum or 10% SRBC.

sacrificed 24 h after the exposure to macrophage toxins and were bled by axillary incision. Blood was collected in chilled tubes, refrigerated for 1 h at 4°C and the sera removed. Enzymatic assays as described in the Materials and Methods section were performed immediately on the fresh harvested sera. Normal mice then received 1.0 ml of the appropriate sera i.p. followed immediately by the i.p. injection of LPS or SRBC. Mice were bled 5 days later and serum antibody titers determined. The i.p. route of injection of both sera and antigen was chosen to avoid circulatory overload and to allow intimate contact *in vivo* between antigen and the enzymatically active sera.

As shown in Table 6, the serum levels of acid phosphatase and LDH activity in mice receiving either macrophage toxin were higher than in control mice. The acid phosphatase values in treated mice were 2-3 times normal while LDH values showed a 12-15 fold increase. The release of acid phosphatase from the lysosomes of phagocytic cells occurs not only as the direct result of cell death, but can be triggered by phagocytosis or after contact with antigen-antibody complexes. However, the cytoplasmic enzyme, LDH, is not secreted or released unless the cell has been destroyed (91). Therefore, measurement of LDH gives a better indication of the amount of cell destruction that has occurred *in vivo*.

Table 6

Serum Enzyme Levels of Normal and Macrophage-impaired Mice[a]

Group	Acid Phosphatase (IU/L)	Lactate Dehydrogenase (IU/L)
Normal[b]	0.53	148
MUS[c]	1.08	1860
Normal[d]	0.50	166
Carrageenan[e]	1.56	2506

[a]Enzymatic assays were performed 24 h after the mice were injected with either PBS, MUS or carrageenan. Each value represents the enzymatic activity of the sera pooled from 50 mice.

[b]Mice were injected i.v. with PBS.

[c]Mice were injected i.v. with 10 mg MUS.

[d]Mice were injected i.p. with PBS.

[e]Mice were injected i.p. with 5 mg carrageenan.

Figures 14, 15 and 16 show the anti-LPS and anti-SRBC titers of mice receiving normal or treated sera with standard doses of antigen. No observable differences were seen in the antibody titers between recipients of either type of serum in any of the groups. Although enzymatic analyses showed macrophage autolysis and release of intracellular enzymes, the increased enzymatic activity and/or the release of soluble factors from damaged macrophages did not affect the in vivo antigenicity of either LPS or SRBC.

Secondary response of normal and macrophage-impaired mice to LPS

It was shown previously that MUS or carrageenan-treated mice responded with a primary humoral antibody response equally as well or better than normal mice to the T-cell independent LPS antigen. It was of interest to determine if a secondary antibody response could be induced as efficiently in mice receiving the primary antigenic dose during macrophage impairment as in normal mice.

Mice were treated with the standard dose of MUS or carrageenan and 6 h later received the priming dose of LPS (1.0 ug or 0.1 ug) i.v. A standard secondary dose of LPS (1.0 ug) was administered 21 days later. Control mice received an equal volume of PBS by identical routes of

Fig. 14. Primary antibody responses of mice to a 1.0 ug dose of LPS given after receiving sera from normal and macrophage-impaired mice. Mice were injected at time 0 with PBS, normal mouse sera or macrophage impaired mouse sera i.p. followed immediately by the injection of 1.0 ug of LPS i.p. Mice were bled 5 days later and titers were determined by passive hemagglutination. Each value represents the antibody titer of sera pooled from 5 mice/ group.

[a]Mice received only antigen.

[b]Mice received antigen and 1.0 ml PBS.

[c]Mice received antigen and 1.0 ml of sera obtained from mice treated with PBS 24 h earlier.

[d]Mice received antigen and 1.0 ml of sera obtained from mice treated with 10.0 mg of MUS 24 h earlier.

[e]Mice received antigen and 1.0 ml of sera obtained from mice treated with 5 mg of carrageenan 24 h earlier.

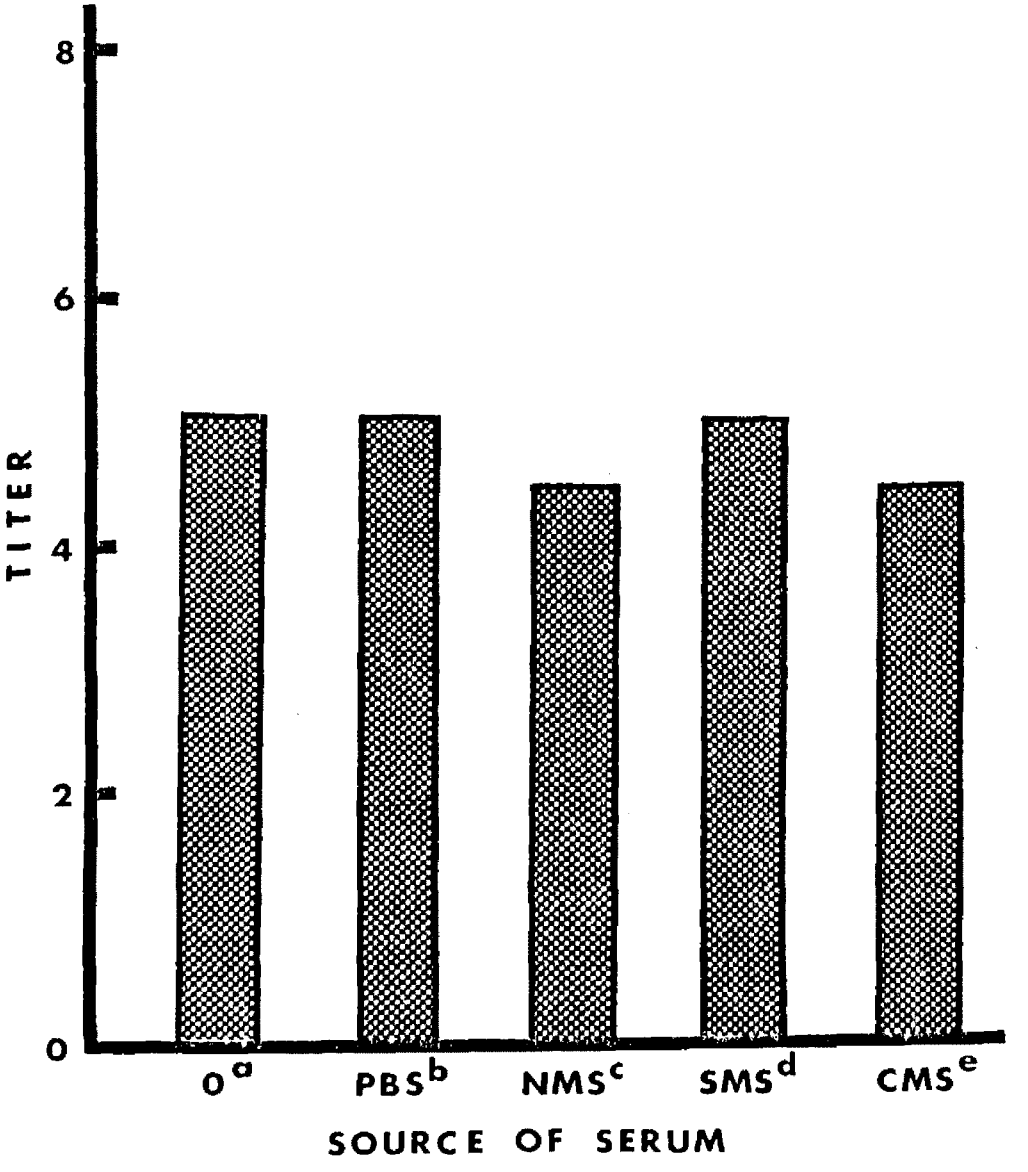


Fig. 15. Primary antibody responses of mice to 0.2 ml of 0.05% SRBC after receiving sera from normal and macrophage-impaired mice. Mice were injected at time 0 with PBS, normal mouse sera or macrophage-impaired mouse sera i.p. followed immediately by the injection of 0.2 ml of 0.05% SRBC i.p. Mice were bled 5 days later and titers were determined by hemagglutination. Each value represents the antibody titer of sera pooled from 5 mice/group.

[a]Mice received only antigen.

[b]Mice received antigen and 1.0 ml PBS.

[c]Mice received antigen and 1.0 ml of sera obtained from mice treated with PBS 24 h earlier.

[d]Mice received antigen and 1.0 ml of sera obtained from mice treated with 10.0 mg of MUS 24 h earlier.

[e]Mice received antigen and 1.0 ml of sera obtained from mice treated with 5 mg of carrageenan 24 h earlier.

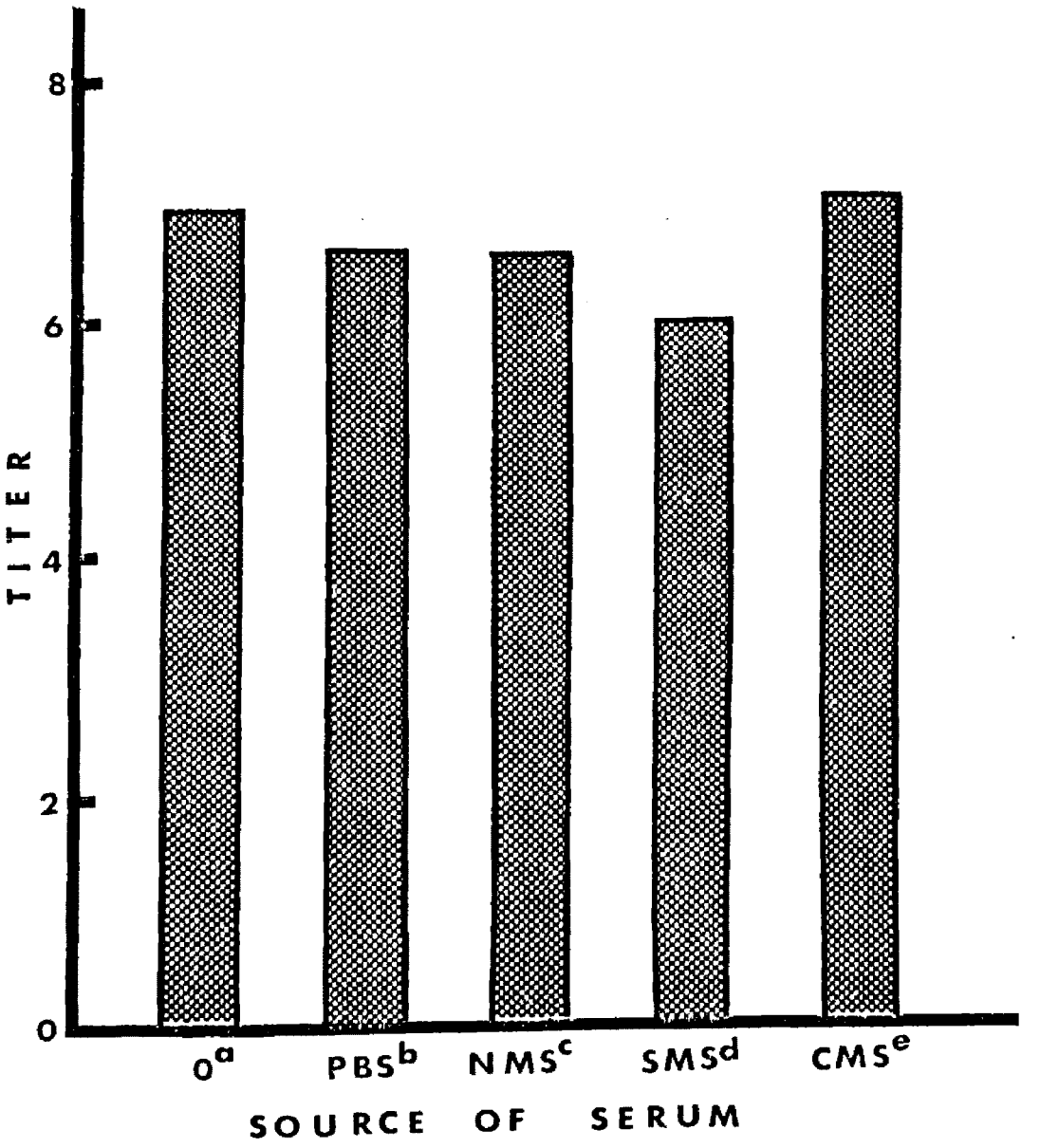


Fig. 16. Primary antibody responses of mice to 0.2 ml of 10% SRBC after receiving sera from normal and macrophage-impaired mice. Mice were injected at time 0 with PBS, normal mouse sera or macrophage-impaired mouse sera i.p. followed immediately by the injection of 0.2 ml of 10% SRBC i.p. Mice were bled 5 days later and titers were determined by hemagglutination. Each value represents the antibody titer of sera pooled from 5 mice/group.

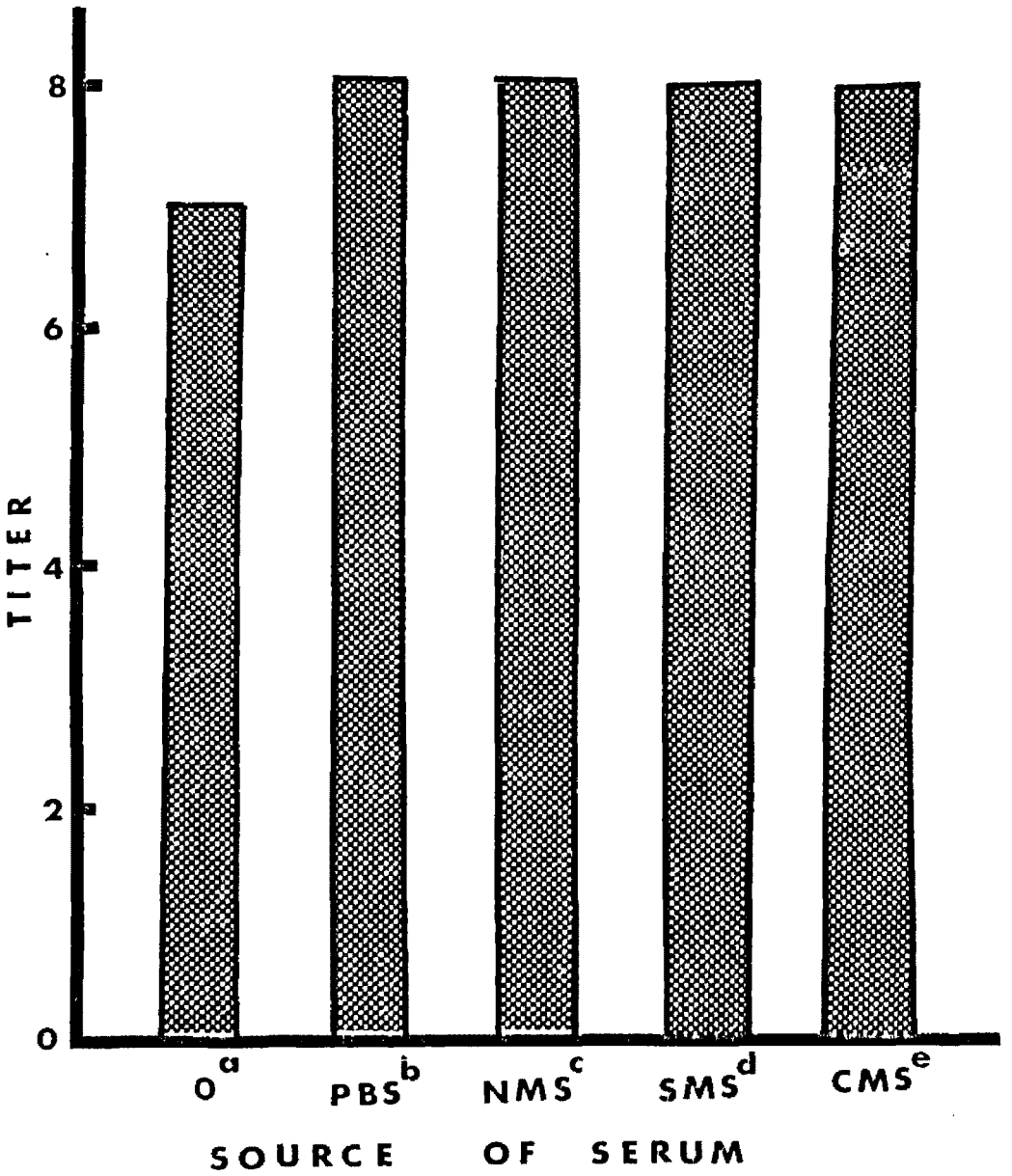
[a]Mice received only antigen.

[b]Mice received antigen and 1.0 ml PBS.

[c]Mice received antigen and 1.0 ml of sera obtained from mice treated with PBS 24 h earlier.

[d]Mice received antigen and 1.0 ml of sera obtained from mice treated with 10.0 mg of MUS 24 h earlier.

[e]Mice received antigen and 1.0 ml of sera obtained from mice treated with 5 mg of carrageenan 24 h earlier.



injection. Additional controls included mice receiving only the primary or secondary dose of antigen. All mice were bled 4 days after receiving the secondary antigen and their sera were pooled and titered.

As seen on Tables 7, 8, 9 and 10, there was no difference in the ability to elicit a secondary antibody response in mice primed with antigen during macrophage impairment as compared with normal mice. The magnitude of the secondary responses to both doses of priming antigens were equal in macrophage-impaired and normal mice. No residual antibody titer was detected following treatment of the sera with 2-ME, thus indicating that the secondary response generated in both treated and normal mice was predominately an IgM antibody response.

Of most interest, however, were the results generated within the control groups. The anti-LPS titers were considerably higher in mice treated with macrophage toxins and given no primary dose of LPS prior to the secondary injection of antigen than in normal mice. The antibody responses generated in the macrophage-impaired group approached the magnitude of the secondary antibody responses of normal mice which received two doses of LPS. Qualitatively, these antibodies to LPS were demonstrated to be of the IgM type. Thus, mice only exposed to either MUS or carrageenan 21 days earlier responded to the single dose of

Table 7

Secondary Antibody Responses of Normal and MUS-treated Mice Primed with 1.0 ug LPS

Group	Treatment[a]	Primary Injection[b] (Day 0)	Secondary Injection[c] (Day 21)	Antibody Titer[d] (Day 25)	
				Total	2-ME Resistant[e]
A	PBS	LPS	LPS	11.5	0
B	MUS	LPS	LPS	11.5	0
C	PBS	None	LPS	6.5	0
D	MUS	None	LPS	10	0
E	PBS	LPS	None	4	0
F	MUS	LPS	None	5	0

[a]Mice were treated at time 0 with 10 mg MUS or PBS i.v.

[b]Mice received 1.0 ug of LPS i.v. 6 h after treatment.

[c]Mice received 1.0 ug of LPS i.p.

[d]Each value represents the antibody titer of sera pooled from 5 mice/group.

[e]Each value represents the antibody titer of sera pooled from 5 mice/group after treatment with 2-ME.

Table 8

Secondary Antibody Responses of Normal and MUS-treated Mice Primed with 0.1 ug LPS

Group	Treatment[a]	Primary Injection[b] (Day 0)	Secondary Injection[c] (Day 21)	Antibody Titer[d] (Day 25)	
				Total	2-ME Resistant[e]
A	PBS	LPS	LPS	11	0
B	MUS	LPS	LPS	11.5	0
C	PBS	None	LPS	4	0
D	MUS	None	LPS	12	0
E	PBS	LPS	None	1	0
F	MUS	LPS	None	4	0

[a]Mice were treated at time 0 with 10 mg of MUS or PBS i.v.

[b]Mice received 0.1 ug LPS i.v. 6 h after treatment.

[c]Mice received 1.0 ug of LPS i.p.

[d]Each value represents the antibody titer of sera pooled from 5 mice/group.

[e]Each value represents the antibody titer of sera pooled from 5 mice/group after treatment with 2-ME.

Table 9

Secondary Antibody Responses of Normal and Carrageenan-treated Mice Primed with 1.0 ug LPS

Group	Treatment[a]	Primary Injection[b] (Day 0)	Secondary Injection[c] (Day 21)	Antibody Titer[d] (Day 25)	
				Total	2-ME Resistant[e]
A	PBS	LPS	LPS	10	0
B	Carrageenan	LPS	LPS	11	0
C	PBS	None	LPS	5.5	0
D	Carrageenan	None	LPS	10	0
E	PBS	LPS	None	4	0
F	Carrageenan	LPS	None	3	0

[a]Mice were treated at time 0 with 5 mg Carrageenan or PBS i.p.

[b]Mice received 1.0 ug of LPS i.v. 6 h after treatment.

[c]Mice received 1.0 ug of LPS i.p.

[d]Each value represents the antibody titer of sera pooled from 5 mice/group.

[e]Each value represents the antibody titer of sera pooled from 5 mice/group after treatment with 2-ME.

Table 10

Secondary Antibody Responses of Normal and Carrageenan-treated Mice Primed with 0.1 ug LPS

Group	Treatment[a]	Primary Injection[b] (Day 0)	Secondary Injection[c] (Day 21)	Antibody Titer[d] (Day 25)	
				Total	2-ME Resistant[e]
A	PBS	LPS	LPS	9	0
B	Carrageenan	LPS	LPS	10	0
C	PBS	None	LPS	6	0
D	Carrageenan	None	LPS	9.5	0
E	PBS	LPS	None	0	0
F	Carrageenan	LPS	None	2	0

[a]Mice were treated at time 0 with 5 mg Carrageenan or PBS i.p.

[b]Mice received 0.1 ug of LPS i.v. 6 h after treatment.

[c]Mice received 1.0 ug of LPS i.p.

[d]Each value represents the antibody titer of sera pooled from 5 mice/group.

[e]Each value represents the antibody titer of sera pooled from 5 mice/group after treatment with 2-ME.

LPS in a manner similar to normal mice receiving both primary and secondary doses of antigen. In summary, then, it was shown that the antibody responses generated in mice receiving antigen 21 days after exposure to MUS or carrageenan, resembled a secondary antibody response.

Due to the consistent finding that secondary-type antibody responses could be elicited by a single dose of antigen in mice exposed to macrophage toxins 21 days prior to immunization, it was of interest to examine the spleens of these animals in order to determine the number of specific antibody forming cells. Groups of mice were treated with either MUS or carrageenan and after 21 days received a single injection of LPS. Five days after immunization the spleens were removed, single cell suspensions were prepared, and the number of direct plaque forming cells (PFC) determined as described in Materials and Methods. Blood samples were also collected from these mice and were tested for hemagglutinating antibody titers.

In normal mice which received both a primary and secondary antigenic dose, the number of direct PFC/spleen was about 25 times higher than that of normal mice which received only one immunizing dose of LPS (Table 11). Therefore, normal mice were primed to LPS with the primary injection of antigen, and a specific population of anti-LPS memory cells generated. These observations were also reflected in the antibody titers. Normal mice which

Table 11

Antibody Responses of Normal and Macrophage-impaired Mice to LPS

Group	Treatment[a]	Primary Injection[b] (Day 0)	Secondary Injection[c] (Day 21)	Antibody Titer[d] (Day 25)		PFC/Spleen[f] (Day 25)
				Total	2-ME Resistant[e]	
A	PBS (i.v.)	LPS	LPS	10	0	13,860
B	PBS (i.p.)	LPS	LPS	10.5	0	13,275
C	PBS (i.v.)	None	LPS	4.5	0	495
D	MUS	None	LPS	9.5	0	5,400
E	PBS (i.p.)	None	LPS	5.0	0	526
F	Carrageenan	None	LPS	10.5	0	4,950
G	PBS (i.v.)	None	None	0	0	14
H	MUS	None	None	0	0	28
I	PBS (i.p.)	None	None	0	0	23
J	Carrageenan	None	None	0	0	27

[a]Mice were treated at time 0 with either 10 mg MUS i.v. or 5 mg carrageenan i.p. Control mice received an equal volume of PBS by identical routes of injection.

[b]Mice received 1.0 ug of LPS i.v. 6 h after treatment.

[c]Mice received 1.0 ug of LPS i.p.

[d]Each value represents the antibody titer of sera pooled from 5 mice/group.

[e]Each value represents the antibody titer of sera pooled from 5 mice/group after treatment with 2-ME

[f]Spleens from mice of each group were pooled and tested for direct plaque-forming cells (PFC).

received only a primary antigenic dose, developed circulating antibody titers of 4.5, whereas, normal mice which received both a primary and secondary antigenic dose developed titers of 10.5.

The number of direct PFC/spleen found in mice treated with either MUS or carrageenan 21 days prior to receiving the only injection of LPS was approximately 10 times that of normal mice receiving only one injection of antigen. Titers of the circulating antibody level revealed that mice treated with macrophage toxins 21 days prior to the primary injection of antigen developed antibody titers that were over 50 fold higher than those observed in normal mice which received only one dose of antigen. Thus, the increased level of circulating antibodies within the mice exposed to macrophage toxins was equivalent to the antibody levels found in normal mice, which had received both a primary and secondary injection of LPS. It is of interest that the circulating antibody titers were equivalent, but the number of direct PFC/spleen was approximately three times lower in macrophage impaired mice than in normal mice. Approximately 13,000 direct PFC/spleen were found in normal mice during the typical secondary antibody response, whereas, only about 5,000 direct PFC/spleen were found in the macrophage-impaired mice during the secondary-type antibody response. Thus, it appears that either the

rate of antibody synthesis is increased in mice previously exposed to macrophage toxins or else a major amount of antibody production has occurred in other lymphoid organs.

Secondary antibody response of normal and macrophage-impaired mice to SRBC

Due to the difficulty in eliciting an IgG type secondary antibody response to the LPS antigen, it was of interest to determine what type of secondary response would be elicited to the SRBC antigen. Experimental protocol was identical to the secondary antibody response studies of LPS, except that mice received a primary immunizing dose of 0.2 ml of a 10% SRBC suspension i.v. followed 21 days later with the secondary immunizing dose of 0.2 ml of 10% SRBC suspension i.p.

Mice which received the primary SRBC antigenic dose during macrophage impairment were capable of eliciting a secondary antibody response to SRBC. This response was both quantitatively and qualitatively equal to the secondary antibody responses of normal mice (Table 12). Only antibodies that were resistant to 2-ME were detectable in all the groups of mice tested, indicating that antibodies of the IgG type predominated. Therefore, the capability of the immune system to prime for a secondary antibody response to the SRBC antigen was not impeded in mice exposed

Table 12

Secondary Antibody Responses of Normal and Macrophage-impaired Mice to SRBC

Group	Treatment[a]	Primary Injection[b] (Day 0)	Secondary Injection[c] (Day 21)	Antibody Titer[d] (Day 25)	
				Total	2-ME Resistant[e]
A	PBS (i.v.)	SRBC	SRBC	11.5	11.5
B	MUS	SRBC	SRBC	11	11
C	PBS (i.p.)	SRBC	SRBC	11	11
D	Carrageenan	SRBC	SRBC	10.5	10.5
E	PBS (i.v.)	None	SRBC	6	3
F	MUS	None	SRBC	8	0
G	PBS (i.p.)	None	SRBC	5.5	3
H	Carrageenan	None	SRBC	7	0
I	PBS (i.v.)	SRBC	None	8.5	8.5
J.	MUS	SRBC	None	9	6
K	PBS (i.p.)	SRBC	None	8	8
L	Carrageenan	SRBC	None	8	6

[a]Mice were treated at time 0 with either 10 mg MUS i.v. or 5 mg carrageenan i.p. Control mice received an equal volume of PBS by identical routes of injection.

[b]Mice received 0.2 ml of a 10% SRBC suspension i.v. 6 h after treatment.

[c]Mice received 0.2 ml of a 10% SRBC suspension i.p.

[d]Each value represents the antibody titer of sera pooled from 5 mice/group.

[e]Each value represents the antibody titer of sera pooled from 5 mice/group after treatment with 2-ME.

to macrophage toxins prior to receiving the primary antigenic dose.

Both control and treated mice possessed residual antibodies to SRBC 25 days after receiving only the primary immunizing dose. Within the control mice these antibodies were determined by 2-ME resistance to be of the IgG type. Although the predominant antibody detectable within the macrophage-impaired mice was also IgG there were, in addition, 2-ME sensitive antibodies indicating that the production of IgM antibodies were prolonged in the macrophage-impaired groups.

The primary antibody responses in mice which received antigen 21 days after exposure to either macrophage toxin were of considerable interest. Normal mice responded to the primary immunizing dose of SRBC with both IgM and IgG antibodies in approximately equal proportions. Mice which were exposed to a single dose of either MUS or carrageenan, 21 days prior to immunization with SRBC, while making quantitatively a primary level response, produced only an antibody response of the IgM type.

In summary, mice exposed to macrophage toxins prior to receiving a primary dose of SRBC were as capable as normal mice in the capacity to prime for a secondary antibody response to 0.2 ml of a 10% SRBC suspension. Residual antibody titers 25 days after exposure to antigen were found to be only IgG in normal mice, whereas macrophage-impaired

mice still possessed some IgM as well as IgG type antibodies. If a single dose of SRBC was given 21 days following exposure to either MUS or carrageenan, only an IgM antibody response was induced, whereas normal mice responded with both IgM and IgG immunoglobulins under similar conditions.

Secondary antibody responses of normal and macrophage-impaired mice to varying priming doses of SRBC

Further experiments were performed to determine if mice treated with macrophage toxins were as efficient as normal mice in their ability to prime for secondary antibody responses with smaller amounts of SRBC than were tested previously. As seen in Table 13, both mice treated with macrophage toxins and normal mice were primed to similar degrees at all doses of SRBC antigen tested. Typical secondary antibody responses, indicated by a higher titer and/or IgG type antibody, were elicited in both groups given the secondary injection of antigen.

Residual primary antibody titers were detectable only within the groups of mice receiving a primary immunizing dose of 0.2 ml of 0.01% SRBC. These residual antibodies were found to consist of both 2-ME sensitive and 2-ME resistant types in mice receiving either MUS or carrageenan. Normal mice not only had a lower concentration of residual antibodies 25 days after immunization, but the hemagglutti-

Table 13

Secondary Antibody Responses of Normal and Macrophage-impaired Mice to Various Priming Doses of SRBC

Group	Treatment[a]	Primary Injection[b] (Day 0)	Secondary Injection[c] (Day 21)	Antibody Titer[d] (Day 25) Total	2-ME Resistant[e]
1	PBS (i.v.)	SREC (0.01%)	SRBC	7	5.5
2	MUS	SRBC (0.01%)	SRBC	10	7
3	PBS (i.p.)	SRBC (0.01%)	SRBC	7.5	6
4	Carrageenan	SRBC (0.01%)	SRBC	9	7
5	PBS (i.v.)	SRBC (0.01%)	None	3	0
6	MUS	SRBC (0.01%)	None	5	3
7	PBS (i.p.)	SRBC (0.01%)	None	2	0
8	Carrageenan	SREC (0.01%)	None	6	4
9	PBS (i.v.)	SREC (0.001%)	SRBC	7	5
10	MUS	SREC (0.001%)	SRBC	7	5
11	PBS (i.p.)	SREC (0.001%)	SRBC	8	5
12	Carrageenan	SREC (0.001%)	SRBC	7.5	5
13	PBS (i.v.)	SRBC (0.001%)	None	0	0
14	MUS	SRBC (0.001%)	None	0	0
15	PBS (i.p.)	SRBC (0.001%)	None	0	0
16	Carrageenan	SRBC (0.001%)	None	1	0
17	PBS (i.v.)	SREC (0.0001%)	SRBC	6.5	3
18	MUS	SREC (0.0001%)	SRBC	6	4
19	PBS (i.p.)	SREC (0.0001%)	SRBC	7	4
20	Carrageenan	SREC (0.0001%)	SRBC	7	4
21	PBS (i.v.)	SREC (0.0001%)	None	0	0
22	MUS	SREC (0.0001%)	None	0	0
23	PBS (i.p.)	SREC (0.0001%)	None	0	0
24	Carrageenan	SREC (0.0001%)	None	0	0
25	PBS (i.v.)	None	SRBC	5	0
26	MUS	None	SRBC	6	0
27	PBS (i.p.)	None	SRBC	5	0
28	Carrageenan	None	SRBC	6	0

[a]Mice were treated at time 0 with either 10 mg MUS i.v. or 5 mg carrageenan i.p. Control mice received an equal volume of PBS by identical routes of injection.

[b]Mice received 0.2 ml of the indicated SREC suspensions i.v.

[c]Mice received 0.2 ml of a 0.05% SRBC suspension i.p.

[d]Each value represents the antibody titer of sera pooled from 5 mice/group.

[e]Each value represents the antibody titer of sera pooled from 5 mice/group after treatment with 2-ME.

nation properties were destroyed after 2-ME treatment, indicating that these were IgM antibodies. Therefore, mice which had received the primary immunizing dose of SRBC during macrophage impairment maintained a higher concentration of residual antibodies as well as antibodies of both the IgG and IgM classes.

Thus, priming for secondary antibody responses to the particulate SRBC antigen was not suppressed if mice received priming doses of antigen during impairment of macrophage phagocytosis. Interestingly, if mice were immunized (0.2 ml of 0.01% SRBC) during macrophage impairment both IgG and IgM classes of antibodies persisted 25 days later. However, in normal mice only IgM antibodies were present. Thus, the quantity and quality of antibody remaining in circulation 25 days after a primary injection of antigen appeared to be dependent upon the dose of antigen and the number of functional macrophages at the time of immunization. More antibody in total and especially more of the IgG class was produced and maintained in mice which had been injected with MUS or carrageenan.

CHAPTER IV

DISCUSSION

In recent years there have been several hypotheses concerning the possible role of macrophages in the induction of humoral antibody responses. Derived from these hypotheses were suggestions that macrophages caused effects ranging from complete abrogation to an acceleration of the antibody response (25, 27, 47, 83). In the present study, the role of the macrophage in humoral immunity was evaluated in vivo. This was done by testing the immune response of mice in which macrophages had been impaired by macrophage toxins. Mice which received the macrophage toxins prior to immunization were still capable of producing normal to supranormal humoral antibody responses to T-cell independent (LPS) and T-cell dependent (SRBC) antigens. However, when the primary antibody response to the T-cell dependent antigen was compared between normal and macrophage-impaired mice, a relationship was observed which was dependent on the dose of antigen. This relationship appeared to affect not only the kinetics and magnitude of the antibody response but also the class of antibody produced. In addition, typical secondary antibody responses could be elicited from mice primed with antigen during macrophage impairment. Moreover, the macrophage toxins themselves appeared to be capable of priming mice for

specific secondary responses to LPS. Thus, it appeared that macrophages might have a role in modulating the immune response *in vivo* by controlling the antigenic dose and promoting long term stimulation rather than being absolutely necessary for "processing" the antigen.

Impairment of in vivo phagocytic activity

Although there was no method available which would deplete completely the mice of macrophages it had been well established that silica and carrageenan were capable of selectively injuring and destroying substantial numbers of macrophages (2, 17). Numerous investigators have employed these agents, both *in vivo* and *in vitro*, to dissect the afferent and efferent limbs of humoral as well as cell-mediated immunity (8 52, 73, 92). However, despite the studies on immunological phenomenon in animals treated with silica or carrageenan, relatively little data had been accumulated on the degree and duration of impaired phagocytic activity *in vivo*. Most commonly, the i.p. route of injection had been used for giving the macrophage toxins to test animals (40, 60, 92). It was possible that materials given by this route of injection could have damaged only the wandering mononuclear phagocytic cells and left the fixed macrophages intact. Dissemination of the i.p. injected toxin was considered to be dependent upon the ingestion of the cytotoxic particles by macrophages in

route to other areas of the body. Persal and Weiser (60) were able to demonstrate disseminated silica particles within the spleen, liver and bone marrow of mice following the repeated i.p. injection of high concentrations of silica. However, no attempt was made to correlate the dissemination of silica particles with the reduction in phagocytic activity *in vivo*.

Impairment by MUS. In the present studies, when various regimens of MUS were given to mice by the intraperitoneal route, essentially normal carbon clearance values were obtained. Since mice were exposed repeatedly to daily i.p. injections of MUS, it was felt that a sufficient amount (total dose of 250 mg) and time (6 days) was allowed for any dissemination to have occurred. The dissemination of MUS with resulting macrophage destruction was not detected by either *in vivo* carbon clearance studies or at autopsy. Large quantities of MUS were found to be sequestered within the peritoneal cavity. Although microscopic examination was not performed, the MUS grossly appeared to be encased in a fibrous-like tissue membrane, often with adhesions to the liver, mesentery and peritoneal membranes. It had been well documented that silica-treated macrophages released a fibroblast stimulating factor (14, 49) which might have been responsible for the rapid sequestering of MUS given by the intraperitoneal route; this could allow for a quick

recovery of the RES.

The localization of MUS observed within the peritoneal cavity and the essentially normal carbon clearance data indicated either that dissemination of a sustained cytotoxic concentration of MUS into areas where it could exert its effect on *in situ* macrophages did not occur, or else the release of MUS was slowed to a rate at which the host could compensate for its cytotoxic effects. In either case, the functional phagocytic capacity of the RES within the animal was not suppressed.

The ability of MUS injected intravenously to suppress the *in vivo* carbon clearance capacity of the RES correlated well with the results of other investigators (52). In the present study, RES suppression was observed by 2 h after mice were given i.v. doses of MUS. With a single treatment, the phagocytic capacity of MUS-treated mice remained suppressed for 4 days. Levy (52) had demonstrated previously that peritoneal mononuclear cells, harvested from mice treated with silica by the i.v. route, showed impaired phagocytosis of SRBC *in vitro*. Similarly in this study mononuclear cells, which were adherent to plastic and were obtained from the peritoneal cavity of mice treated with MUS i.v. 24 h earlier, were less efficient in their ability to phagocytize *C. stellatoideae* cells *in vitro*. Thus, data were obtained from phagocytic studies *in vivo* and *in vitro* that the i.v. injection of MUS was capable of

impairing the phagocytic activity of the fixed as well as the wandering macrophage populations.

Since MUS was not metabolized *in vivo*, it was thought that it could be used as a long term macrophage toxin. That is to say, after ingestion of the particle the macrophage would undergo autolysis releasing the toxic particle to be rephagocytized by other cells. This process would be repeated until the host could sequester the MUS in a fibrous or in a granulomatous lesion. However, this study demonstrated that the suppressive effect of i.v. administered MUS on phagocytosis was maintained for only 4 days. Therefore, it appeared that the toxic MUS particles were sequestered from the host rather quickly and the cytotoxic action either abolished or compensated for by the host. Regeneration of a functional macrophage population, capable of yielding normal *in vivo* carbon clearance values, occurred within 5 days. Hence, a single injection of MUS could be used as a selective cytotoxic agent to evaluate the inductive phage of immunological responses which occurred in less than 5 days, but this treatment could not chronically suppress macrophage function *in vivo* without repeated injections.

In summary, the MUS preparation employed within this study was capable of impairing the phagocytic capacity of the RES. It was found that the i.p. route of injection for this macrophage toxin, even with multiple injections of high

doses (50 mg/day for 5 days) did not suppress the ability of the RES to remove carbon from the peripheral blood. Conversely, the i.v. administration of a single dose of MUS was found to suppress the ability of the RES to clear carbon as early as 2 h after its administration. Maximum impairment of phagocytic activity was obtained 6 h after the i.v. administration of MUS and this impairment was maintained for 4 days. From analysis in vivo and in vitro, it was shown that a substantial number of the phagocytic cells of the RES had been destroyed or impaired. From the above data, it was concluded that MUS could be used to analyze the role of macrophage phagocytosis in the induction of the immune response in vivo.

Impairment by carrageenan. Carrageenan, a high molecular weight polygalactose extracted from sea algae, was also used as a macrophage toxin in this study. It had been shown to exert its toxic effect by inducing changes in the permeability of the lysosomal membrane resulting in autolysis of the phagocytic cell (2, 17).

Carrageenan was capable of suppressing the phagocytic activity of the RES when administered by either the i.v. or i.p. routes. However, mice would tolerate an i.v. dose of only 1 mg carrageenan. A solution of carrageenan was quite viscous even at low concentrations and the viscosity alone could have caused the deaths observed in mice which

received 2 mg. Indirect evidence suggested that dissemination of the carrageenan occurred very rapidly throughout the host when the toxin was given i.p. Data in support of this, were from observations that suppression of the RES was observed within 2 h following i.p. injection of carrageenan.

Concurrently with the reduced carbon clearing capacity of *in situ* macrophages, it was also found that the wandering macrophages harvested from the peritoneal cavity of mice treated with carrageenan were less effective in their ability to phagocytize *in vitro*. Therefore, as was found with MUS, carrageenan was also found to be capable of impairing the phagocytic activity of the fixed as well as the wandering macrophage populations.

Changes in the serum enzyme concentrations of mice treated with either macrophage toxin also indicated that cell destruction had occurred. Weissman et al. (91) had shown previously that up to 25% of certain acid hydrolases were released from the lysosomes of phagocytic cells during phagocytosis. Hence, increased serum levels of enzymes normally contained within the lysosomes of phagocytes might not necessarily reflect cell destruction. Therefore, in this study both the lysosomal enzyme, acid phosphatase, and the cytoplasmic enzyme, LDH, were measured. Both enzymes were found to be elevated in mice exposed to macrophage toxins. The concomitant increase of both acid

phosphatase and LDH indicated that the enzyme levels found in the peripheral blood were due to cell autolysis.

In addition, two other assay systems were used to demonstrate the in vivo cytotoxicity of MUS and carrageenan. The first system employed radiolabeled LPS injected into normal and mice treated with the macrophage toxins. The tissue distribution of LPS found in normal animals correlated well with the results of other investigators (11, 12, 54, 68). It was demonstrated in this study that over 75% of the LPS was found in the liver of normal mice, while less than 1% was associated with any of the other tissues assayed. On the other hand, less LPS was found in the livers of mice treated with macrophage toxins and large amounts of LPS were retained in the peripheral blood of macrophage-impaired mice. Higher levels of LPS were consistently found in the spleens of macrophage-impaired mice than were found in normal mice. Thus, the treatment of mice with macrophage toxins had impaired the ability of the macrophages to phagocytize not only particulate materials, such as carbon or yeast cells, but also the colloidal antigen (LPS) which was subsequently tested for immunogenicity in these mice.

The other auxiliary system used to demonstrate RES impairment was a measurement of the sensitivity of macrophage-impaired mice to the toxicity of LPS. It had been well established that agents which impaired the phagocytic

activity of the RES increased the sensitivity of impaired animals to the toxic effects of LPS (6, 20). The results obtained in this study indicated that mice treated with MUS or carrageenan were respectively, 40 and 3000 times more susceptible than normal mice to the lethal effects of LPS.

Thus, by numerous independent assay systems this study showed that both MUS and carrageenan under controlled conditions were capable of impairing the phagocytic capacity of the RES in a reproducible and consistent manner. This suppression was not permanent with either toxin, but it persisted for only 4 days. Maximal suppression of RES activity occurred 6 h after mice received either toxin and persisted at this level for at least 24 h and 48 h after treatment with MUS and carrageenan, respectively. Therefore, it was felt that if mice were exposed to antigen 6 h after treatment with either macrophage toxin any role the macrophage might play in the afferent limb of humoral antibody formation should be altered. If phagocytosis of antigen was a prerequisite for the induction of an optimal immune response *in vivo* then mice receiving antigen during impaired phagocytosis should either (1) not be able to respond with antibody production, (2) respond with antibody production but, at a sub-optimal level, or (3) show a delayed antibody response until a competent macrophage

population was regenerated.

Primary antibody responses

In other investigations (8, 52, 72, 80) it was observed that primary antibody responses could be suppressed in animals treated with agents which impaired the RES if the impairment occurred prior to immunization. Thus, it was inferred that phagocytosis, processing, and/or presentation of antigen to lymphocytes by macrophages were essential events for the induction of optimal antibody responses. This study did not demonstrate any suppression of the primary antibody response to either LPS or SBRC antigens regardless of the time at which mice were exposed to macrophage toxins in relation to the antigenic dose. The only observable difference between macrophage-impaired mice and normal mice was the increased primary antibody response of the impaired mice to low immunizing doses of the SRBC antigen. This increased antibody response was induced only when mice were immunized 6 h and 24 h after exposure to macrophage toxins. Interestingly, this was the period during which the phagocytic capacity of the RES was most severely suppressed. Thus, it appeared that a decreased functional macrophage population *in vivo* could be correlated with the increased antibody response demonstrated in this study.

Keller (48) had shown with antibody responses generated *in vitro* that alteration of the macrophage/lymphocyte ratio

could modify mitogen-induced lymphocyte proliferation. A macrophage/lymphocyte ratio of ten macrophages per one lymphocyte consistently blocked proliferation and even a ratio of one macrophage per one lymphocyte was inhibitory. Conversely, ratios of one macrophage per 25 or 100 lymphocytes resulted in an increased proliferative response. Since both MUS and carrageenan have been shown to be selectively toxic for macrophages and not lymphocytes (2, 17) it could be assumed that the macrophage/lymphocyte ratio was decreased in treated mice. Thus, it appeared that there may have been a direct competition between macrophages and lymphocytes for antigen or that the macrophages suppressed proliferation of antigen-stimulated lymphocytes in normal mice.

When the tissue distribution of radiolabeled LPS was quantified, consistently higher than normal amounts of LPS were found within the spleens of macrophage-impaired mice. Similarly, others (75, 76) have demonstrated an increased localization of chromium-labeled SRBC within the spleens of macrophage-impaired mice. It appeared that if circulating antigen was not removed and sequestered by macrophages, it might be capable of binding directly to splenic lymphocytes.

In other investigations (8, 52, 72) it had been demonstrated that the alterations in primary antibody responses

of macrophage-impaired mice were correlated with both the routes of macrophage toxin administration and antigen presentation. Bice et al. (8) demonstrated normal to enhanced splenic PFC when SRBC antigen was given i.v. to mice treated previously with carrageenan i.p. However, when SRBC antigen was given i.p. to mice treated similarly with carrageenan, the number of splenic PFC was reduced greatly. Other investigations (80, 81) also had demonstrated a decreased number of splenic PFC when both carrageenan and SRBC antigen were given i.p. It was concluded that the cytotoxic action of carrageenan was confined to the peritoneal cavity and did not affect macrophages throughout the animal. This allowed intravenously administered antigen to be processed by the unaltered functional splenic macrophages. In opposition to the above data, the results of the present study indicated that carrageenan was not confined to the peritoneal cavity. In fact, its effect was disseminated rapidly throughout the host as evidenced by the impairment of intravenously administered colloidal carbon. An explanation for the decreased responses observed in the previously cited work could be that 24 h following the i.p. injection of carrageenan, the predominant cell type within the peritoneal cavity was PMN. Therefore, the decreased anti-SRBC responses observed could have been due to antigen degradation by the PMN

rather than the lack of macrophage processing.

In summary, other studies have shown that the injection routes of both the macrophage toxin as well as the antigen appeared to be critical in elucidating the role of the macrophage as an effecter cell of humoral immunity. Also recent studies by Benner and Vanoudenaren (7) demonstrated that the systems used for quantitative assessment of the immune response were important when attempting to evaluate the total humoral response. For example, specific PFC were found in substantial numbers in organs not normally assayed such as the bone marrow. The present investigation has demonstrated that primary antibody responses could be induced at normal or a supranormal levels during macrophage impairment. This was evaluated by determinations of the total circulating antibody response. It was felt that measurement of circulating antibody titers truly reflected the immunological status of the whole animal. Furthermore, it appeared that there might have been direct competition between macrophages and other immunologically active cells. This phenomenon was especially evident when macrophage-impaired mice were given a sub-optimal dose of SRBC. Thus, it appeared that macrophages could function to modulate the immune response <u>in vivo</u> by regulating the amount of antigen available for the triggering, rather than by some processing step. It also appeared that by controlling the amount of antigen available for antigenic stimulation, macrophages

might play a major role in influencing the resultant class of antibody synthesized.

Secondary antibody responses

Relatively little data had been obtained from studies in vivo, which evaluated the ability of macrophage-impaired animals to be primed for secondary antibody responses. Stevenson and Stavitsky (79) demonstrated that the primary antibody response to T2 phage could be suppressed with anti-macrophage globulin (AMG). However, this regimen of AMG did not impair the secondary responsiveness of these animals. This indicated that if animals were immunized during impaired macrophage function the priming mechanism for secondary antibody responses was not affected.

Likewise, the present study demonstrated that mice treated with MUS or carrageenan before the primary immunization appeared to develop typical secondary antibody responses when challenged with a second dose of LPS. Since immunization with this regimen of LPS elicited antibodies of only the IgM class, it was impossible to evaluate the secondary response by a change in antibody class. However, the kinetics and the magnitude of the response indicated that a typical secondary antibody response to LPS had been elicited. Mice, primed during macrophage impairment with varying doses of SRBC, responded with typical secondary responses when challenged with a second dose of

SRBC. These antibody responses were characterized by an increased antibody titer and/or by antibody production of the IgG class. Thus, it appeared that priming with LPS or SRBC for a secondary antibody response occurred as efficiently in mice which received the primary immunization during macrophage impairment as in normal mice.

In the course of running controls of the effect of MUS and carrageenan during the time of priming mice for secondary antibody responses, several observations were made. Mice treated with either toxin 21 days prior to immunization with SRBC developed higher than normal primary antibody responses. It was determined that macrophage-impaired mice responded with antibodies of only the IgM class, regardless of the immunizing dose. However, normal mice responded to a high dose of SRBC (0.2 ml of 10%) with both IgM and IgG class antibodies, whereas with a low immunizing dose of SRBC (0.2 ml of 0.05%) only antibodies of the IgM class were generated. This elevation of the antibody response in animals treated with macrophage toxins had been noted by others (33, 42, 46, 62). The present study did not evaluate further the reason for the higher response obtained from mice exposed to macrophage toxins 21 days prior to immunization with SRBC.

More interesting were the observations made when mice were exposed to macrophage toxins 21 days prior to

immunization with LPS. It was noted that a secondary-type antibody response could be elicited with only one dose of antigen. From correlation of hemagglutination and PFC data, it appeared that this response might be due to increased antibody production in committed lymphocytes rather than the development or expansion of a memory cell population. Thus, regulatory factors which controlled the rate of negative feedback mechanism might have been altered by (1) the decreased number of functional macrophages, (2) factors released from the impaired macrophages, or (3) the macrophage toxins themselves.

The possibility that B-cells were derepressed during macrophage impairment or that major antibody production had occurred in organs other than the spleen could not be disregarded. Alternately, the possibility that the MUS and carrageenan preparations employed within this study were contaminated with LPS cannot be excluded totally. However, the fact that (1) anti-LPS titers could never be elicited from mice injected with only MUS or carrageenan, (2) direct anti-LPS splenic PFC were not demonstrated in mice receiving only MUS or carrageenan, (3) similar observations have been noted by other investigators (33) employing different antigens, and finally (4) the probability that both the MUS and carrageenan would be contaminated with the same chemotype of LPS made the possibility of LPS contamination very remote.

As observed in the present study, as well as in other investigations (46), immunization during macrophage impairment appeared to prolong the macroglobulin response to both LPS and SRBC antigens. Consistently higher titers with evidence of IgM class antibodies were detected in macrophage impaired mice 21 days after immunization with a single dose of LPS or SRBC. Thus, especially when the anti-SRBC was evaluated, it appeared that the rate and duration of IgM production might be altered in mice exposed to macrophage toxins prior to immunization. The antibody response observed in normal mice appeared to involve a switch from the early biosynthesis of predominantly macroglobulin antibody to the production of 7 S molecules. Conversely, this switch to 7 S antibody production did not occur as efficiently when mice were immunized during macrophage impairment.

Henry and Jerne (43) had shown that IgM could function as a positive feedback molecule. That is to say, antibody contained within the 19 S fraction when given prior to a moderate dose of SRBC antigen increased the antibody response 10-15 fold. It appeared that the enhancement as well as the prolongation of the primary macroglobulin antibody response observed within this study might be directly related to the decreased functional macrophage population during immunization. Macrophages might function by

controlling the epitope density of antigen available for triggering other cells in the immunological sequence. During macrophage impairment, antigen was not sequestered and, therefore, was available to repeatedly stimulate the immunological active cells. This repeated stimulation might result in the enhanced production of the positive feedback IgM molecule and may explain the enhanced, as well as the prolonged antibody responses observed in this study.

Concluding remarks

The precise mechanism by which macrophages participate in the immune response still remains to be elucidated. From this study it appeared that macrophages may modulate, in vivo, the immune response by controlling the amount of antigen available for direct stimulation of other cells in the immunological sequence. Thus, the quantity as well as the quality of the immune response, may be controlled indirectly by macrophages acting as a sequesterant of antigen and not by increasing directly the immunogenicity of the antigenic molecule via some processing step. These observations were consistent with current studies which indicated that a small amount of antigen remained on the surface of the macrophage and was not catabolized (88). Also, other studies demonstrated that macrophage-associated antigen could stimulate lymphocytes (74, 78, 82). Such a mechamism would insure an economical

response by reserving a small amount of antigen to stimulate the immune system over a prolonged period. Therefore, it appeared that phagocytosis, processing, and presentation of antigen by macrophages to lymphocytes were not prerequisites for the induction of immune responses *in vivo*. The role played by the macrophage would be one of a passive bystander rather than as an active participant.

CHAPTER V

SUMMARY

The effects of macrophage toxins on the immune response of mice *in vivo* were investigated. It was determined that a reduction of the functional macrophage population did not suppress the humoral immune response to either the T-cell independent LPS antigen or the T-cell dependent SRBC antigen.

Two specific macrophage toxins, MUS and carrageenan, were determined to be capable of reducing phagocytic activity *in vivo*. The degree of suppression varied with each toxin and depended on the route of administration. MUS was capable of reducing phagocytic activity *in vivo* only when it was administered i.v. On the other hand, carrageenan reduced the phagocytic activity when administered either i.v. or i.p., although the i.p. route allowed the administration of a larger dose with fewer toxic side effects. The initiation and persistance of maximum impairment of phagocytic activity *in vivo* was similar for both macrophage toxins. Maximum suppression was observed approximately 6 h after the administration of either toxin and recovery to a normal level of phagocytic activity occurred after 5 days. Extensive analysis *in vivo* and *in vitro* by other parameters also indicated that mice exposed

to MUS or carrageenan had impaired phagocytic activity.

The kinetics of the primary immune response to LPS were evaluated in mice treated with macrophage toxins. Mice immunized with LPS during macrophage impairment were capable of producing antibody responses equivalent to those obtained in normal mice. There was no change in either the kinetics or magnitude of the antibody response in mice immunized during impaired macrophage activity. These results suggested that macrophages were not a critical cellular component for the induction of humoral antibody responses to the T-cell independent LPS antigen.

The kinetics of the primary immune response to SRBC were evaluated in mice treated with macrophage toxins. It was demonstrated that mice immunized with SRBC during macrophage impairment were capable of producing antibody responses equivalent to or higher than those obtained in normal mice. Macrophage-impaired mice immunized with a low dose of SRBC (0.2 ml of 0.05% SRBC) generated higher titers and demonstrated circulating antibodies 24 h earlier than normal mice. Macrophage-impaired mice immunized with an intermediate dose of SRBC (0.2 ml of 1% SRBC) responded with the generation of both IgM and IgG class antibodies. Whereas, normal mice responded to this intermediate dose of SRBC with only IgM class antibodies. Thus, the class of antibody produced was dependent upon the immunizing dose

of antigen as well as the functional macrophage population. These results suggested that macrophages were not essential for the induction of humoral antibody responses to the T-cell dependent SRBC antigen, *in vivo*. However, it appeared that macrophages might compete with other cells for the quantity of antigen available for lymphocyte stimulation or that the macrophages suppressed proliferation of antigen-stimulated lymphocytes in normal mice.

Typical secondary antibody responses appeared to have been elicited from mice treated with MUS or carrageenan 6 h prior to primary immunization. Mice primed with varying doses to LPS during macrophage impairment and followed with a standard secondary dose of LPS 21 days later responded with a typical quantitative increase of IgM class antibodies. Mice immunized with varying doses of SRBC during macrophage impairment and 21 days later injected with a standard secondary dose of SRBC responded with a typical quantitative increase of predominately IgG class antibodies. Therefore, a reduction in the number of functional macrophages had not impaired the ability of mice to prime for secondary antibody responses.

In the course of running controls on the effects of MUS and carrageenan on priming for a secondary antibody response several observations were made. Mice treated with either toxin 21 days prior to immunization with SRBC de-

veloped higher than normal primary antibody responses to SRBC. It was determined that macrophage-impaired mice responded with antibodies of only the IgM class. However, the antibody response of normal mice was dependent upon the dose of SRBC administered. Normal mice responded to a high dose of SRBC (0.2 ml of 10% SRBC) with both IgM and IgG class antibodies; with a low dose of SRBC (0.2 ml of a 0.5% SRBC) only antibodies of the IgM class were generated.

More interesting were the observations made when one dose of LPS was given to mice exposed to macrophage toxins 21 days prior to immunization with LPS. It was noted that a secondary type antibody response could be elicited with only one dose of antigen. From correlation of hemagglutination titers and PFC data, it appeared that this response might be due to increased antibody production in committed lymphocytes rather than the development or expansion of a memory cell population. These secondary type antibody responses were not evaluated further.

It was also observed that immunization during macrophage impairment appeared to prolong the macroglobulin response to both LPS and SRBC antigens. Consistently higher titers with evidence of IgM class antibodies were detected in macrophage-impaired mice 21 days after immunization with a single dose of LPS or SRBC. Thus, it appeared that the rate and duration of IgM production might be altered in

mice exposed to macrophage toxins prior to immunization.

In summary, it appeared that macrophages were not critical cellular components of the ARU. It was demonstrated in the present study that the induction of primary antibody responses, as well as the priming for secondary antibody responses, did not appear to be macrophage dependent. From the data obtained it appeared that macrophages might function *in vivo* by sequestering antigen and, thereby controlling the amount of antigen available for activation of other immunologically active cells rather than by increasing the immunogenicity of an antigen by some critical processing event.

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